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Vol. 25, No. 2, September–October, 1960

Russian Original Dated March–April, 1960

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BIOCHEMISTRY

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(Russian Original Dated March-April, 1960)

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THE ORIGIN OF CREATIVE INSPIRATION

(Translated from Biokhimiya, Vol. 25, No. 2, pp. 193-197, March-April, 1960)

The reality of the present day – visual features of communism actively manifested in our country, the strengthening of the people's democratic order in a number of countries in Europe and Asia, the growth of the worker's movement in capitalistic countries, the previously unseen scope of the struggle for national independence in the colonial and dependent countries, the struggle for peace in the entire world – is the triumph of Leninism in economic, social, and political spheres of human activity.

But the scientific genius of V. I. Lenin was not limited only to social sciences, and it will not be an exaggeration to say that the development of natural science at the present stage, in the era of atomic energy and the conquest of space, is no less a triumph of Lenin's philosophical ideas.

The Lenin analysis of the development of physics at the start of our century, and the discovery of the causes of the crises in physics at that time, due to the collapse of one of the old, set, crushing metaphysical concepts on the structure of matter and the nature of the microworld surrounding us, had an enormous, basic value for the future development of science.

On the basis of a deep philosophical analysis of the revolution which gave birth to this crisis in natural science, V. I. Lenin raised and solved anew the most important problems in natural science, answered the new philosophical problems raised by the revolution in natural science, and delivered a smashing blow to idealism in science.

Of exceptional importance is the position of V. I. Lenin on the development of objective truth, and on the dialectical character of this development. V. I. Lenin teaches that objective truth is a complex dialectical process which is realized in the course of contradictions, their emergence, development, and destruction. Specific forms of these contradictions are the relative and absolute aspects of truth, which constantly interact as if they were opposite sides and tendencies. V. I. Lenin showed that objective truth exists as a process of motion from relative truths through a medium of relative truth to absolute truth, the most important factor of this process being practice.

The Lenin theory of knowledge lighted the way for scientific development in the entire epoch ahead. The magnificient results of this path were the development of the solar and earth sputniks, actual prospects for interplanatary flights, and the development of new uses for atomic energy. And it is not an accidental, but a completely natural development that these discoveries occurred in the country of Leninism.

The philosophical studies of Lenin have enormous meaning for all other natural scientific disciplines, including biology. Lenin's evaluation of Darwinian doctrines, his statements on questions of the origin of life and other important problems of biology had and have a fundamental value for scientific development of these problems. The entire development of biology shows that dialectical materialism is the basis for creating new principles and approaches for explaining the conformity in the development of organisms and the remaking of the constitution of living bodies. These principles received wide reflection in the Michurian studies. All this is related fully to the youngest branch of biology, biochemistry.

Biochemistry, the study of the chemical laws of the formation and conversion of living matter, was formed as an independent scientific discipline at the end of the past century, when the physicochemical basis of life, the study of the organic forms of the motion of matter, according to the statement of F. Engels, was in the very primary stage of development.

Having been enriched by the experience of physics and chemistry and by achievements in physiology, biochemistry from the very beginning revealed the qualitative uniqueness of the chemical activity of life's phenomena and confirmed their materiality. The synthesis by organic chemists of certain important compounds which enter into the composition of living organisms had a very important meaning for the confirmation of materialistic concepts in natural science. In this connection, F. Engels wrote (Ludwig Feuerbach and the End of Classical German Philosophy) that chemical substances produced in animal and vegetable organisms remained "things in themselves" until organic chemistry began to synthesize them one after another and thus the "thing in itself" was converted to "things for us". The achievements in chemistry delivered a death blow to idealism and its more dangerous variation vitalism.

Together with this, the achievements in organic chemistry included a demonstration of the danger of the simplistic concept of life. And actually, the main accomplishment in the development of biochemistry at this early stage was that the study of matter of a biological origin broke away from the study of the processes causing such matter's emergence and conversion. From here on, the entire picture being developed as a result

of such investigations carried a static character, however, and was immobile and set.

However, as the chemical nature of the various biological processes was unravelled, there was greater verification of the idea of movement, development, and the dialectical concept of metabolism as a unity of contradictions – assimilation and dissimilation, movement and dormancy, constancy and variability.

Thus, it was established that for an organism to assimilate various substances which are ingested as food and for their incorporation into the composition of tissues and organs, the necessary stage is the disintegration of these substances, the catabolism of protein into amino acids, of fats into glycerin and fatty acids, of complex carbohydrates into monosaccharides. On the other hand, in a complete organism, the catabolism of a series of compounds, particularly of carbohydrates, occurs through the stage of phosphorylation, which is characterized by the complication of the structure of the substances.

V. I. LENIN

90th Anniversary of His Birth

The conclusively demonstrated fact of rapidly completed restoration of all substances in the organisms showed that any organ, tissue, or structure in an organism is very quickly and completely restored; these elements consist of other atoms and molecules and demonstrate "just the opposite" of what had been thought. At the same time, each organ, tissue, and structure performs an appropriate function during its life span. Thus, in spite of the high level of the processes of metabolism in nervous tissue which is retained for the life span, the inherent or acquired neuroreflex stereotypes, etc., are retained.

The contradiction of the dynamic and the conservative in metabolism to a great degree is determined, first of all, by the characteristics of the structure and metabolism of two classes of compounds, proteins and nucleic acids.

In the case of these compounds the basis of the variability and plasticity are such factors as the exceptional variability of proteins, their ability to interact with almost all compounds found in an organism, and the diversity of the ways in which the same substances in an organism may be converted.

The conservation of metabolism is determined by the constancy of the qualitative amino acid composition– in spite of all the varieties of proteins, in their composition are revealed, as a rule, only about 20 individual amino acids; species, tissue and organic protein specificities, and species specificities of nucleic acids which display a strictly constant ratio between the purine and pyrimmidine bases. Thus, as biochemistry developed, the fundamental law of the dialectic – the hypothesis of the unity and struggle between contradictions – obtained more and more confirmation.

While interpreting such universally vital functions as respiration, fermentation, nutrition, photosynthesis, and chemical activity of the formation and conversion of various substances in an organism, along with exposing the differences between organisms, the general features in the processes of metabolism occurring in various organisms were more fully revealed. The fact that the investigations were conducted dynamically played an important role in the development of concepts concerning the unity of the biological processes and the functional activity of organisms.

The concept of the unity and completeness of chemical and functional activity is confirmed also in the now solidly established hypothesis of the genetic proximity and close bond between respiration and fermentation, two basic channels along which occurs an influx of energy to the organism; in this whole picture, also, is the nitrogenous metabolism of higher and lower organisms, animal, and vegetable.

When giving a scientific definition of life as a form of existence of protein bodies, Engels emphasized the role of the main factor, i. e., protein. However, in spite of the importance of protein in the vital functions of an organism, it would remain a dead load without the intimate relations which exist between protein and various other compounds. Protein fulfills its main functions in a compound – fermentative, immunologic, and reproductive – with substances of another nature. Thus, in conjunction with vitamins or metals (copper, iron, zinc, cobalt), protein demonstrates the properties of enzymes. Proteins become viruses with the expressed ability to self-reproduce only by being part of the composition

of nucleoprotein; the separate components of nucleoprotein, proteins and nucleic acids, do not have this ability.

The formation of the proteins themselves is accomplished with participation of RNA, whereas all synthetic processes in the organism occur only with participation of enzymes – catalytically active proteins. These are additional examples of the relation and interaction of various links in metabolism in an organism and a new confirmation of the Lenin hypothesis that the universal principle of development must "unite, connect, combine with the universal principle of the unity of the world, nature, motion, and matter" (V. I. Lenin, Works, Vol. 38, p. 251).

The Lenin hypothesis of the relation of the part to the whole, of the individual to the whole, has enormous value for a true understanding of the problem of interconnection, unity of analysis and synthesis, and exposure of the character of the bonds in the metabolic process.

"The individual", V. I. Lenin pointed out, "does not exist other than in relation which leads to the general. The general exists only in the individual, through an individual" (V. I. Lenin, Philosophical Notebook, State Press for Political Literature, p. 329, 1947).

From this, the biochemists should definitely conclude that when going along the path of individualization and further separation of cellular elements or multi-enzymic biochemical processes, we simultaneously must always bear in mind the necessity of synthesizing these elements, of perceiving all functions specific to the organism, to the whole.

Studies in recent years show that the basis of biochemical functions is structural and chemical heterogeneity, the interaction of multi-enzymatic systems deployed in various submicroscopic structures. This structural and enzymatic interaction assures the development of a biochemical function within the cell. The development of a vital function orginates on the basis of chemical and structural heterogeneity and biochemical qualitative diversity. Each structure and each element within the cell differ from each other both in their composition and in the potentialities of the functional activity within them. A clear example of such a process is the synthesis of protein.

Biochemical data show that protein synthesis in a living cell occurs as a result of the interaction of various cellular elements. In the soluble fraction of a cell, fermentative activation of amino acids, occurs after which there is a transport and transmission of these amino acids to a soluble ribonucleic acid, the origin of which, apparently are the nuclei. The next important stage in this synthesis is the transfer of the amino acids from molecules of ribonucleic acid to a structure of ribonucleoprotein of microsomes. The mitochondria, being, so to speak, power stations of the cell, supply the necessary energy for synthesis of the peptide bond of the proteins.

What obtains generally here is the function of protein synthesis which exists in the individual, i. e., in those cellular elements where subsequently or simultaneously are accomplished the individual, interconnected processes which lead to the creation of a given biochemical function.

Rejecting the notion of the eternity of life and also the notion of the instantaneous origin of life on the earth, dialectical materialism teaches that life, being a special form of the motion of matter, is the product of matter's development and increasing complexity. Life is not eternal; it emerged at a definite historical stage as a result of long development, when and where favorable conditions were created. Having exposed the idealistic state of affairs in the thought of Ernst Mach (Machism), V. I. Lenin indicated that "organic matter is the latest phenomenom, the fruit of continuous development" (V. I. Lenin, Works, Vol. 14, p. 63). Modern natural science completely refutes the idealistic concepts of the instantaneous origin of life, the spontaneous generation of life, and the mechanistic concepts of life originating directly from inorganic matter. It proves with complete irrefutability that life is the result of the long development of matter from the lowest to the highest, from the simple to the complex.

Modern natural science looks upon life on earth as the result of the development of the earth itself, of the complexity of matter, of the origin of life from substances of an inorganic nature.

Criticizing the viewpoints of the Russian Machist, Bogdanov, V. I. Lenin noted that "the study of the science of the structure of matter, of the chemical composition of food, of the atom and electron, can become obsolete and does become obsolete with each day, but the truth cannot become obsolete, so that man cannot be nourished by ideas alone or bear children by one platonic love" (V. I. Lenin, Works, Vol. 14, p. 173). Underlining ours-Ed.).

When V. I. Lenin wrote these lines the prevailing point of view on biochemistry was that proteins, fats, and carbohydrates were the main and almost the only constituents of food; the value of mineral elements, and especially the role of essential vitamins, was still unknown. Presently, the role mineral components in an organism is more deeply understood, and with each year the number of vitamins, the necessary ingredients of healthy and full-valued food, increases.

The achievements of modern biochemistry confirm the materialistic hypothesis of the inexhaustibility of matter and its infinity, of the new horizons being opened before scientists as the movement of science advances. It is sufficient to recall that the chemical activity of the respiration and fermentation processes had been deciphered, and that the reaction systems by means of which the accumulation and transfer of energy in biological objects are achieved have been discovered. Significant successes have also been attained in the study of

the chemical activity of photosynthesis; ideas have been formed concerning the multi-enzymatic systems and these ideas are the foundation for studying the interconnection of the metabolic processes; the chemical structure and the biochemical role have been determined for a number of biologically important compounds (hormones, vitamins). Researchers have learned to isolate the individual structural elements of a cell, to characterize the anatomy of molecules of biologically important substances. An important feature in the development of biochemistry in its present stage is the determination of the structure and mechanism of biosynthesis of those compounds which play a leading role in the most universal processes of metabolism. All this opens the broadest perspectives in the further development of biochemistry.

In the future, we shall see investigation of the biochemical functions of proteins and nucleic acids, particularly the structure and mechanism of the action of the "active grouping of enzymes", hormones of a protein nature, toxins, antibodies, and other physiologically active substances. The final stage of these investigations should be the synthesis of proteins which posses immunological, catalytic, and hormonal activity. Synthesis of biologically active proteins will open a new stage in the development of contemporary biochemistry and will create the prerequisites for developing new methods of chemical engineering based on methods of synthesizing various chemical compounds which are more economical than the presently existing methods.

The greatest tasks of contemporary biochemistry are the deciphering of the chemical activity of life's phenomena, the disclosure of the chemical activity of disorders which are the basis of pathological processes, including those which are the result of the effect on an organism of various physical factors (ionizing radiation, factors related to space flights, etc.).

A very essential question of both theoretical and practical importance arises in connection with the study of the problem of photosynthesis. Solving the mystery of photosynthesis will yield unlimited opportunity for increasing the productivity of horticulture and the creation of new forms of organisms.

Leninism looks upon practice as the criteria of truth. Science ceases to exist when it loses the force to bind theory with practice and science with production, the bond being the basic condition for the development of any branch of knowledge. Practice should be wide, international, and not restricted to narrow frameworks.

At the present stage, the progress of biochemistry has opened wide possibilities of employing biochemical mechanisms in the interest of practice, in the interest of the society. What has been discussed above, which is far from a complete list of the problems which biochemistry is now close to solving, testifies to the great and constantly increasing information offered by practical biochemistry, especially valuable for those branches of practical activity of man in which it is necessary to deal with live objects: in medicine, agriculture, in industries reprocessing raw material of a biological origin. The Seven-Year Plan for the development of the national economy opens great opportunity for a successful utilization of the achievements of contemporary biochemistry in the construction of the communist society.

And if in the creation of an imposing picture of a biochemical universe we obvioulsy see the efficiency of the Lenin ideas, then there can be no doubt about the fact that in the future advance of science, Marxist-Leninist study will become all the more the tested weapon of naturalists, and Soviet biochemistry, constructed on the foundation of a dialectical materialistic world outlook, will make its worthy contribution to world science.

A STUDY OF THE PHOSPHORUS COMPOUNDS IN THE DEVELOPMENT OF Azobacter vinelandii USING P^{32}

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(Translated from Biokhimiya Vol. 25, No. 2, pp. 198-210, March-April, 1960)
(Original article submitted June 30, 1959)

It has been shown in many studies that polyphosphates are used in the biosynthesis of proteins and nucleic acids of some lower organisms, supplying phosphorus and energy for these processes [1-10]. It is also possible that polyphosphates can play an important energetic role in the process of nitrification [11]. A number of results indicate that polyphosphates are synthesized from orthophosphates by intermediate formation of some acid soluble phosphorus compounds [1, 12, 13]. There is evidence to show that both the synthesis and expenditure of polyphosphates occurs with the participation of ATP [1, 14-17].

In previous work [5, 6] we studied the quantitative changes which occurred with some phosphorus compounds, including polyphosphates, and suggested a path for their synthesis and utilization during the development of azobacter. This work gave further details on the processes of biosynthesis and utilization of polyphosphates in the cell of azobacter and showed the interrelation of polyphosphate metabolism with that of other phosphorus compounds, especially with nucleic acids.

We had previously observed the presence in the acid soluble fraction of azobacter along with the nucleic acids, of some phosphorus components, part of which absorbed in the ultraviolet [6]. Similar facts were found in microorganisms by other investigators [18, 19]. In our work, using the method of ion exchange chromatography, we tried to separate the "excess phosphate" of the RNA fraction into a number of individual phosphorus components, so as to study their properties and rate of metabolism of P^{32}.

METHODS

In order to enlarge the study of the phosphorus compounds we used a culture of A. vinelandii grown on a nitrogen-free Burke medium (on molecular nitrogen) and on the same medium in the presence of ammonia nitrogen. The seeded culture was grown for two days in the corresponding medium which contained P^{32} (in the form of $K_2HP^{32}O_4$ in the amount of 0.5 m curie/liter), carefully washed with culture medium which did not contain P^{32}, and transferred to a new nonradioactive medium on which further growth of the azobacter occurred. The conditions of culturing the azobacter and growth of the material for analysis were described earlier [6].

For an explanation of the interrelation of the metabolism of the polyphosphates with nucleic acids we used a special type of experiment. The azobacter cells were first deprived of phosphorus for 20-24 hours by growing them in a nitrogen-free Burke medium which lacked this element, and then were transferred to a new medium which contained 25% above the normal of P and P^{32} (0.5 m curie/liter $K_2HP^{32}O_4$). Enrichment of the cells with phosphorus was carried out for one hour and the sample was collected for analysis every 15 min. The azobacter cells, carefully washed from P^{32}, were placed in the same medium, but without phosphorus, and after 20, 40, 60, and 180 min samples were taken for analysis. The material was fixed by freezing the water suspension of the bacteria at low temperature.

In the study of the rate of metabolism of P^{32} of different phosphorus compounds of A. vinelandii we used the method and calculation of coefficients as described in previous papers [5, 6].

For calculation of the specific activity (SA) of P^{32} of the various phosphorus compounds we determined the orthophosphate immediately or after 7 and 30 min hydrolysis with 1 N HCl or after calcination with concentrated $HClO_4$. Orthophosphate in the form of phosphomolybdic acid was removed from the solution with a mixture of isobutanol-benzene (1 : 1). In one portion of the extract we determined P by reaction with $SnCl_2$ [12, 20]; in another, after evaporation of the solvent in air we measured the radioactivity with a Geiger-Muller counter (end counter of the type SI 2-B). We introduced a correction in the calculation of activity for the decay of P^{32} [21]. Measurement of the radioactivity of ribonucleotide P was carried out directly on the paper [20] after electrophoretic separation of the RNA hydrolyzate [22]. The rate of exchange of RNA-P^{32} was determined by summating the phosphorus radioactivity of the four nucleotides which composed it. Measurement of the DNA-P radioactivity was carried out in preparations of the Na salts obtained after preliminary complete separation of them from the bacterial mass of the soluble nucleotides and RNA [23].

Ion exchange chromatography of the acid soluble mononucleotides and phosphorus components of the RNA fraction was carried out on a column with Dowex-1 (× 10; 200-400 mesh, formate form, dimensions of column 18 cm × 1 cm^2; rate of passing solution 0.5 ml/min; volume of fraction 5 ml, washing volume 500 ml). We used the formicacid system described by Hurlbert et al., [24] in the modification of Gilbert and Yemm [25] (Figs. 1 and 2). The acid soluble nucleotides were separated from the TCA extract in the form of Ba salts by the process previously described [6]. The acid soluble RNA fraction, which contained besides the RNA nucleotides the "excess" phosphorus components, was obtained by the modified method of Schmidt and Tannhauser [6, 26]. The individual components of the eluted fractions were identified by the values of their absorption at different wave lengths [24]. Measurement of P^{32} radioactivity in the separate eluates was run directly in 2 ml of solution using a specially constructed target.

In the experiments carried out with P^{32} it was very important to know the character of the calculation of the activity and the time of uptake of P^{32} in the separate phosphorus compounds. When the radioactive material was grown on a medium without P^{32}, the SA itself could not show the intensity of functional activity of the given compound in the cell. However, the degree of decrease of activity could indicate the amount of synthesis of the given compound because of the nonradioactivity of the orthophosphate medium. The activity of the definite phosphorus compound calculated on 1 mlrb of the cell takes into account the factor of dilution of P^{32} when the outer orthophosphate enters the cell. The activity of the phosphorus compound calculated on a biomass of 1 ml of culture medium considers the factor of distribution of P^{32} between the cells newly formed by division. In order to gain some idea of the amount of synthesis of one or another of the phosphorus compounds in the bacterial cell and the character of the exchange of P^{32} between them, it was important to carry out three types of calculation of the activity on μ g P, on the cell, and on the biomass*), otherwise a change in the rhythm of cell division in the cycle of the bacteria would make interpretation of the results unreliable [27].

A special study showed that during development of the cultures on the nonradioactive medium the loss of P^{32} from the cells did not occur [28, 29]. Some fall in SA of total phosphorus in the bacterial cells with growth of the culutres explains the loss of P^{32} from the measured cells [30].

RESULTS AND DISCUSSION

Comparison of the activity of different phosphorus compounds in seeded cultures of A. vinelandii grown on nitrogen free media and on ammonia nitrogen in the presence of P^{32} showed a considerable increase in P^{32} activity in cultures with suppression of nitrification and the same time of uptake of P^{32} (Tables 1 and 2). This is evidently connected with the fact that addition to the medium of even the minimum amount of N leads to considerable hastening of the exchange of P^{32} of almost all the phosphorus compounds of the cell, and expecially of RNA [28, 31].

The activity of phosphorus metabolism becomes still more evident if we turn to the dynamics of quantitative accumulation of some phosphorus compounds in the cycle of development of azobacter, depending on nitrogenous components of the medium [5]. Growing azobacter on ammonia nitrogen leads to suppression of nitrification and to a sharp increase in amount of RNA. Due to this higher level of RNA in the culutres grown on ammonia nitrogen there is a more rapid and intense tempo of their development compared to the nitrifying cultures [32].

The strong drop at the beginning of the logarithmic phase of development in the SA of most of the phosphorus compounds in cultures with suppression of nitrification, as compared to nitrifying cultures, depends on the more intense tempo of cell division in the first (Tables 1 and 2). Hence the nitrogenous components of the medium have great value for the quantitative content of phosphorus compounds and for the rate of exchange of their phosphorus in the cells.

The activity of orthophosphate in the process of development of azobacter on a medium without radioactive phosphorus in strongly lowered. This is because in the process of development of cultures there is an intensive assimilation of the orthophosphate of the medium and its utilization in the synthesis of organic phosphorus compounds (Table 1). Among the acid soluble organic phosphorus compounds of the seeded cultures the highest SA occurs in the mononucleotides and especially in the di- and triphosphates (Table 1).

We should mention that P_{labile} of the acid soluble mononucleotides of A. vinelandii separated by ion exchange chromatography are 60-70% ADP and ATP phosphorus (Fig. 1). As Fig. 1 shows, the SA of these compounds greatly surpasses the SA of other nucleosides and triphosphates of this fraction. This permits us conditionally to designate the acid soluble di- and triphosphates of the nucleotides as the ATP-ADP system. The SA of the P_{labile} acid soluble nucleotides in the seeded cultures reaches the level of activity of orthophosphate (Table 1). In calculation on the biomass the fall in activity of orthophosphate and soluble polyphosphate in the latent phase of development is accompanied by an increase in activity of mononucleotides and especially the phosphate esters of carbohydrates, so that the process of phosphorylation of carbohydrates occurs with participation of nucleoside di- and triphosphates which are markedly activated in the latent phase (Table 1).

Thus, the polyphosphates can be used in phosphorylation of carbohydrates and nucleotides which in turn share in formation of nucleic acids, proteins, polysaccharides, etc. Other investigators have observed a similar effect [15, 33, 34].

It is known that acid insoluble polyphosphates are synthesized faster and have greater physiological activity than the soluble one. The first synthesis of polyphosphates

* The biomass is the weight of bacteria (μ g) which occupy a unit volume of culture medium.

TABLE 1. Change in P^{32} Activity of Phosphorus Compounds in the Acid Soluble Fraction during Growth of A. vinelandii

Age of cultures, hours	Orthophosphate			Soluble polyphosphate			Mononucleotides						Phosphate esters of carbohydrates			P_{total}, acid soluble		
							P_{labile}			P_{total}								
	I	II	III	I	II	III	I	II	III	I	II	III	I	II	III	I	II	III
On molecular nitrogen																		
Starting	360	720	864	240	374	449	400	84	101	210	155	186	150	134	161	265	1383	1660
5	206	558	670	220	110	132	270	94	113	176	278	334	128	294	353	174	1241	1490
10	130	296	355	0	0	0	220	106	243	130	377	452	94	296	355	116	970	1162
15	84	152	228	0	0	0	306	184	276	156	234	351	68	324	486	82	710	1065
24	80	136	306	Trace	Trace	Trace	342	178	400	160	196	441	64	178	400	79	510	1147
36	41	64	186	69	76	220	320	100	290	150	146	423	50	136	396	66	422	1225
48	31	60	189	70	124	390	166	38	120	108	76	243	53	51	164	57	308	986
On ammonia nitrogen																		
Starting	428	392	470	280	388	465	458	82	98	278	222	266	186	157	182	287	1150	1383
4	134	390	468	216	162	194	320	96	115	196	259	311	208	292	350	172	1105	1325
8	106	268	322	166	74	89	356	136	163	162	280	336	144	274	329	135	897	1076
12	36	85	204	55	11	26	216	110	264	70	204	490	56	144	346	52	426	1066
24	38	41	163	0	0	0	230	126	567	76	139	625	30	80	360	46	287	1148
30	40	28	130	65	66	299	221	62	280	75	102	460	39	71	320	54	268	1209
45	32	28	138	70	108	540	92	18	86	60	50	249	42	37	185	54	222	1112

Notes: I) SA in counts/min/μg P; II) counts/min in 1 mlrb of cell (SA × g P of the given compound in 1 mlrb of cell); III) counts/min in biomass (SA × μg P of the given compound in the biomass of 1 ml of medium).

occurs in the form of acid insoluble high molecular weight compounds, from which the second are formed as comparatively low polymers [3, 12, 13, 35]. A number of authors [1, 12, 13] have suggested that some acid soluble phosphorus compounds are precursors in the synthesis of polyphosphates in the cell.

In the latent phase (Tables 1 and 2) the synthesis of insoluble polyphosphates and nucleic acids occurs chiefly

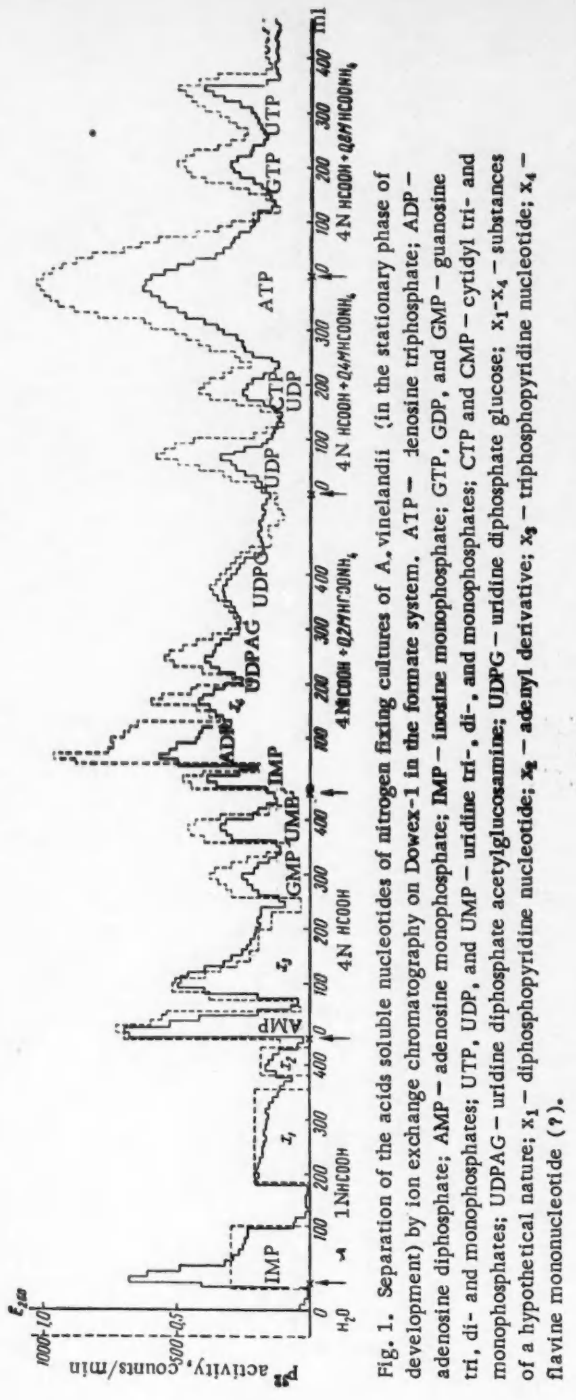

Fig. 1. Separation of the acids soluble nucleotides of nitrogen fixing cultures of A. vinelandii (in the stationary phase of development) by ion exchange chromatography on Dowex-1 in the formate system. ATP – adenosine triphosphate; ADP – adenosine diphosphate; AMP – adenosine monophosphate; IMP – inosine monophosphate; GTP, GDP, and GMP – guanosine tri-, di- and monophosphates; UTP, UDP, and UMP – uridine tri-, di-, and monophosphates; CTP and CMP – cytidyl tri- and monophosphates; UDPAG – uridine diphosphate acetylglucosamine; UDPG – uridine diphosphate glucose; x_1-x_4 – substances of a hypothetical nature; x_1 – diphosphopyridine nucleotide; x_2 – adenyl derivative; x_3 – triphosphopyridine nucleotide; x_4 – flavine mononucleotide (?).

from orthophosphate of the external medium with participation of the phosphorylation of carbohydrates and nucleoside di- and triphosphates which in the given time interval almost double their activity. Upon this, the synthesis in all probability occurs through the ATP-ADP system.

Tables 1 and 2 show that the fall in activity of the polyphosphates calculated on the biomass is as a rule accompanied, in the logarithmic phase of development, by an increase in activity of the acid soluble nucleotide fraction which shows the possibility of transfer of P (and energy) through just the compounds of this fraction. The most marked fall in activity of acid insoluble polyphosphates in the logarithmic phase of development indicates their share in the synthetic processes of the cell.

These results show at a glance that not only the formation, but the utilization of polyphosphates occurs with participation of the ADP-ATP system which is a universal donor of energy for various synthetic processes. It is not impossible that the use of polyphosphates can also take place by stepwise degradation accompanied by liberation of energy. Thus, we can conclude that polyphosphates through the ADP-ATP system phosphorylate nucleoside di- and monophosphates and their derivatives which are the prosthetic groups of many enzyme systems.

In the stationary phase of development (Table 2) the slowing of the rate of synthetic processes leads to decreased rate of utilization of acid insoluble polyphosphates and to transfer of some of their parts to lower molecular weight forms, the acid soluble polyphosphates (in Tables 1 and 2 we compare the activity of soluble and insolbule polyphosphates in 30 and 45 hours on NH_4^+ and also in 36 and 48 hours on N_2). Thus, the soluble polyphosphates in azobacter have a secondary origin, being formed by depolymerization of the acid insoluble polyphosphates.

When we compare the exchange of RNA-P^{32} and the acid soluble mononucleotides in the latent phase of development we observe the same level of their SA up to 8-10 hours of development (Tables 1 and 2). This fact is evidently not accidental, but is a consequence of the interrelation of metabolism of soluble nucleotides and RNA. Actually, the polynucleotides formed from mononucleotides can serve in equal degree as sources for the formation of components of the soluble mononucleotide fraction [36, 37]. In the first half of the logarithmic phase of development there is the most energetic decrease in activity of RNA-P^{32}, accompanied by a rise in P^{32} activity of acid soluble mononucleotides. This is an indication that RNA can take part in the biosynthesis of proteins also through the components of the nucleotide fraction which forms with amino acids compounds of the type of acyladenylates [38, 39].

The data on the rate of exchange of P^{32} of the RNA mononucleotides of A. vinelandii during development are given in Table 3. This shows that the relative SA of mononucleotides P^{32} from RNA during the whole life cycle of A. vinelandii corresponds to molar proportion of the nucleotides which compose it. This fact evidently indicates the metabolic equivalence of all the RNA molecules in the process of development of azobacter. Our results corresponds to recently published work [40, 41].

The data of Table 2 indicate the parallelism between rate of cell division and amount of dilution of the SA of DNA-P^{32} from nonradioactive orthophosphate of the medium. It is interesting that with doubling the population there is a twofold decrease in radioactivity of DNA-P, calculated on

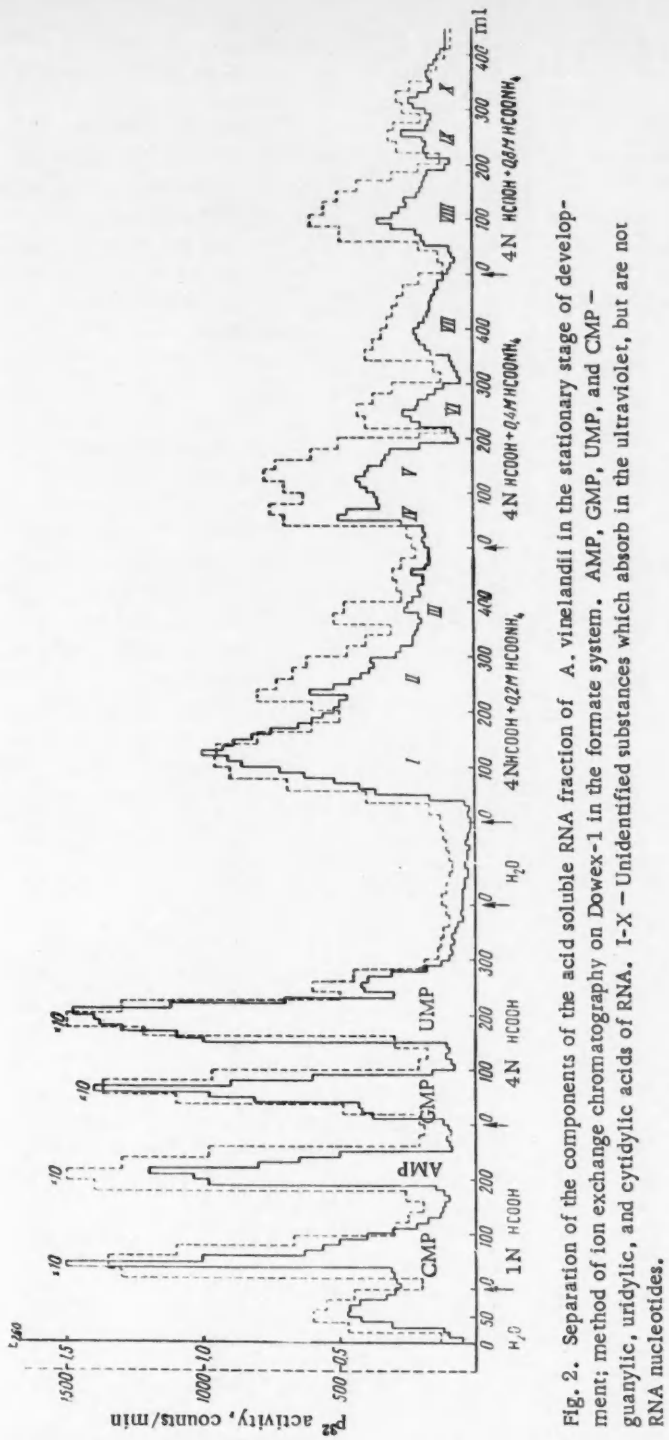

Fig. 2. Separation of the components of the acid soluble RNA fraction of A. vinelandii in the stationary stage of development; method of ion exchange chromatography on Dowex-1 in the formate system. AMP, GMP, UMP, and CMP – guanylic, uridylic, and cytidylic acids of RNA. I-X – Unidentified substances which absorb in the ultraviolet, but are not RNA nucleotides.

the cells. This shows that before the beginning of division about twice the amount of DNA is synthesized in the cell [32, 36, 42].

When we consider the activity of DNA-P^{32} calculated on the biomass (Table 2) we see that the exchange of DNA-P with other phosphorus compounds (including with RNA-P) does not occur or is especially slight. Also, as is clear from the same table, DNA-P as compared to RNA-P, has much lower radioactivity, which changes very slightly in the life cycle of the cell when calculated on the biomass. This can indicate the relative metabolic stability of DNA.

At the present time there is much discussion of the question of the use of polyphosphates in the synthesis of

TABLE 2. Change in P^{32} Activity of Phosphorus Compounds of the Acid Insoluble Fraction in the Growth of A. vinelandii

Age of cultures, hours	mlrb of cells/ml medium	Insoluble polyphosphate			RNA-P			Excess phosphorus of RNA fraction			DNA-P			Phospholipid P			Phosphorylated polysaccharides of bacterial residue			P_{total} of bacteria		
		I	II	III	I	II	III	I	II	III	I	II	III	I	II	III	I	II	III	I	II	III
On molecular nitrogen																						
Starting	1,2	380	878	1054	214	1085	1302	116	575	690	144	166	200	176	470	634	100	110	132	186	4670	5602
5	1,2	324	972	1166	174	1134	1361	114	490	588	126	162	195	170	500	675	91	77	92	149	4650	5580
10	1,2	302	997	1196	156	1232	1478	110	464	557	101	162	195	162	524	707	57	28	34	143	4540	5450
15	1,5	204	732	1098	115	937	1406	106	378	567	86	132	198	96	484	726	—	—	—	95	3400	5100
24	2,25	194	302	680	80	562	1264	64	282	634	74	82	190	106	218	716	25	34	77	79	2180	4908
36	2,9	80	150	435	66	412	1195	60	236	684	60	66	191	108	366	1061	—	—	—	64	1670	4850
48	3,2	35	86	275	64	356	1121	54	254	800	50	59	189	124	234	1052	—	—	—	62	1476	4724
On ammonia nitrogen																						
Starting	1,2	309	1236	1484	280	1694	2033	194	904	1085	140	192	230	188	502	602	120	132	158	200	5750	6900
4	1,2	316	1255	1507	180	1746	2095	166	846	1015	106	200	240	182	538	646	112	94	113	148	5750	6900
8	1,2	267	1243	1492	158	1810	2072	110	868	1032	92	202	244	168	546	657	80	38	46	124	5500	6600
12	2,5	122	525	1314	54	795	1908	56	309	742	47	83	200	56	282	677	25	48	115	48	2408	6020
24	4,0	95	269	1156	48	395	1778	42	197	788	31	46	208	50	150	675	32	43	172	43	1487	5950
30	4,5	41	213	960	45	360	1620	40	170	765	33	46	208	54	173	778	44	48	216	36	1288	5800
45	5,0	36	156	751	46	327	1574	38	156	749	33	44	210	67	182	874	58	52	250	35	1120	5600

Note: Designations the same as in Table 1.

proteins and nucleic acids [1-10]. Comparison of the data of Table 2 on P^{32} activity of insoluble polyphosphates and RNA in the process of development of azobacter unfortunately does not give an exact answer as to the possibility of use of polyphosphates in the synthesis of RNA. In the latent and logarithmic phases of development there occurs a fall in SA of P^{32} both of the insoluble polyphosphates and the RNA to about the same extent (Tables 2 and 4). To explain the very important question of the possibility of transfer of P from polyphosphate to RNA requires specially set up experiments. The data in Table 4 show conclusively that enrichment of the azobacter cells with P in the presence of P^{32} followed by impoverishment in this element leads to transfer of part of the polyphosphate P into RNA. This is clear from the sharp decrease in SA of P^{32} of insoluble polyphosphates, accompanied by a rise in the SA of P^{32} of RNA, upon which this transfer of P occurs through P_{labile} of soluble mononucleotides (ATP-ADP system).

Under the conditions of enriching the cell with phosphorus (Table 4) the formation of RNA and polyphosphates can occur apparently by two independent processes. The possibility of use of polyphosphates in the synthesis of nucleic acids and proteins in the absence of P in the medium has been shown by many investigators [2, 7-9, 12, 17, 43]. Demonstration of the use of polyphosphates for the synthetic needs of the cell is given by the recent work of Sall, Mudd, and Takagi [4], carried out with synchronously dividing cultures of C. diphtheriae [4].

These facts indicate that polyphosphates, under definite conditions of culturing the microorganisms can

TABLE 3. Specific Activity and Relative Specific Activity of the Phosphorus of RNA Mononucleotides during Development of A. vinelandii on Moleuclar Nitrogen

Age of cultures, hours	Guanylic acid		Adenylic acid		Cytidylic acid		Uridylic acid		RNA	
	I	II	I	II	I	II	I	II	I	II
Starting	260	100	205	100	219	100	172	100	214	100
5	211	81	167	82	179	82	140	81	174	81
10	189	73	149	72	163	74	125	72	156	72
15	140	54	110	53	118	53	93	54	115	53
24	98	38	76	37	82	37	64	37	80	37
36	80	31	63	30	68	31	53	30	66	31
48	78	30	61	30	65	29	52	30	64	30
Mole % mononucleotides [26]	1,26		1,00		1,06		0,80		—	
Relative SA of mononucleotides (SA of adenylic acid equals 1)	1,27		1,00		1,07		0,84		—	

Note: I) SA in counts/min/μ g P; II) relative SA in %.

TABLE 4. Content of Different Forms of Phosphorus and Their Specific Activity in A. vinelandii

Time, min.	Acid soluble phosphorus										Acid insoluble phosphorus								mlrb of cells in 1 ml of medium
	orthophosphate		soluble polyphosphates		mononucleotides				phosphate esters of carbohydrates		insoluble phosphates		RNA-P		DNA-P		Phospholipid P		
					P_{labile}		P_{total}												
	I	II	I	II	II		I	II	I	II	I	II	I	II	I	II	I	II	
Enriched with phosphorus with P^{32}																			
15	0,40	50	0,30	25	32		0,35	22	0,75	15	0,47	41	3,51	30	1,17	18	1,60	23	2,2
30	0,76	116	0,42	26	80		0,39	31	0,96	27	0,81	120	4,13	77	1,21	20	1,86	25	2,2
45	0,65	187	0,58	27	97		0,42	40	1,34	31	1,65	183	5,32	95	1,24	19	2,13	28	2,2
60	0,76	223	0,72	54	155		0,45	47	1,87	35	1,88	230	6,46	107	1,26	22	2,24	31	2,3
Impoverished with phosphorus without P^{32}																			
20	0,64	187	0,70	57	178		0,48	50	1,80	40	1,50	180	6,50	118	1,25	20	2,18	29	2,3
40	0,53	120	0,66	61	184		0,42	61	1,70	47	0,95	123	6,42	135	1,24	19	2,00	27	2,4
60	0,47	98	0,60	58	206		0,40	58	1,62	52	0,81	84	6,15	146	1,23	19	1,88	25	2,5
180	0,45	103	0,41	45	118		0,36	46	1,55	58	0,60	28	5,68	150	1,20	17	1,50	28	2,8

Note: I) Content of phosphorus compounds in μg P/mlrb of cells; II) SA of phosphorus compounds in counts/min/μg P.

actually be used for the synthesis of proteins and nucleic acids. This can be shown in the development of azobacter either under conditions of phosphorus starvation, when the cells do not have a sufficient amount of P from the external medium (Table 4), or in the period of especially intense cell growth and division, when there is a supplementary mobilization of all the energy reserves (Table 2).

As we have mentioned earlier, we have found in the RNA fraction of azobacter a considerable quantity of phosphorus compounds (excess P of the RNA fraction) [5, 6]. Using the method of ion exchange chromatography, we have separated this excess phosphate into a series of individual phosphorus components, some of which have a high SA, somewhat surpassing the SA of P^{32}, of RNA mononucleotides which indicates in them a great importance for cell metabolism (Fig. 2). The majority of these phosphorus compounds absorb in the ultraviolet part of the spectrum at 220-270 mμ.

The chemical nature of the "excess" phosphorus compounds of the RNA fraction of A. vinelandii has not been specially studied in this work. According to the preliminary results of Stacey et al. [18], these substances give a positive reaction with ninhydrin and are associated in some form with peptide aminoacids. It is possible that some of them are glycerophosphates and inosine phosphates combined with cellular proteins [18, 19].

As is evident from Table 2, the SA of P^{32} of phospholipids in the latent phase of development remains at about the same, rather high level, which shows the absence of perceptible exchange of their P with other phosphorus compounds of the cell during this period of development. The P^{32} SA of bacterial residues, preferably from phosphorylation of polysaccharide residues, is considerably decreased in the latent period, which is a consequence of its use in phosphorylation of some cellular components. At the end of the logarithmic and stationary phases of development, the P^{32} activity of phospholipids and phosphorylated polysaccharides calculated on the biomass rises considerably. This occurs because to the end of the life cycle of the cultures, mineralization of a number of organic phosphorus compounds (including polyphosphates) is accompanied by liberation of P, part of which goes to the phosphorylation of lipids and polysaccharides of the cell, in which form during this period of development there is storage of phosphate.

On the basis of the experimental data obtained previously [6] and in the present work, the path of exchange of the chief phosphorus compounds of azobacter can be given in the following scheme.

This scheme gives further details of phosphorus metabolism in azobacter and corresponds to the chief ideas obtained recently in our laboratory [3, 10, 12, 17].

SUMMARY

The nitrogenous components of the medium have a very important effect on the quantative changes of the phosphorus compounds and on the rate of exchange of their phosphorus during development of A. vinelandii.

In cultures grown on ammonia nitrogen, the quanti-

tative content of the majority of phosphorus compounds and the level of P^{32} activity in them is considerably higher than in nitrifying cultures.

The first synthesis of polyphosphates occurs in the form of acid insoluble phosphorus compounds from which by degradation comparatively low polymers are formed. The synthesis of nucleic acids and insoluble polyphosphates occurs from orthophosphate with intermediate formation of phosphorylated carbohydrates and nucleoside di- and triphosphates of the acid soluble fraction. The polyphosphates are used in the synthesis of proteins and nucleic acids or in the period of especially intensive synthetic activity of the cells (logarithmic phase), or at the moment of phosphorus starvation (absence of phosphorus in the medium). Polyphosphates at definite periods in the development of the culture can be used in phosphorylation of acid soluble carbohydrates and mononucleotides. Both synthesis and utilization of polyphosphates occurs with participation of the ADP-ATP system which is a universal energy donor.

The exchange of P^{32} of RNA and soluble mononucleotides in the azobacter cell is interrelated. This is indicated by the fact that RNA can take part in the synthetic processes through the components of this fraction. The SA of the P^{32} of RNA mononucleotides during the whole life cycle of A. vinelandii corresponds to their molar ratio, which shows the metabolic equivalence of all the RNA molecules.

Doubling of the cell population is accompanied by a comparable decrease in P^{32} activity of DNA. Hence, before beginning of division in the azobacter cell, twice the amount of DNA has accumulated.

The excess phosphate of the RNA fraction of azobacter shows a large number of different phosphours components, some of which have a high exchange rate of P^{32}, which indicates the metabolic activity of these compounds.

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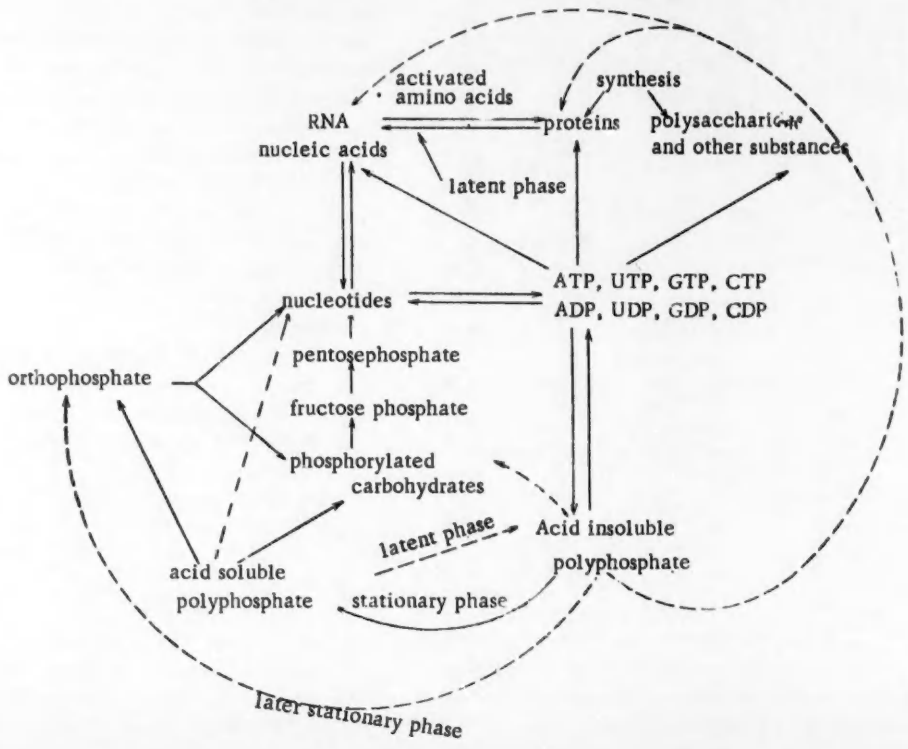
Scheme of Transformations of Phosphorus Compounds of A. vinelandii



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† Original Russian pagination. See C. B. translation.
‡ Original Russian pagination. See English translation.

THE SINGLE STEP POLAROGRAPHIC WAVE OF THE PROTEINASE OF B. SUBTILIS

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It is well known in polarography that proteins in the Brdicka reagent usually show a double step polarographic wave. Rarely, however, in this reagent it is possible to show the existence of a single step wave for proteins. Recently such a wave has been shown for blood serum; its potential peak corresponds to the potential maximum of step I of the protein wave [1].

We have obtained a single step wave of the proteinase of Bacillus subtilis, the half wave potential of which does not correspond to the half wave potential of the first or second step wave of proteins [2]. The half wave potential of the B. subtilis proteinase falls in the field of more positive potential than the half wave potential of proteins which show a single step wave in an ammonia buffer in the absence of cobalt or in a solution of indifferent electrolytes; KCl, NH_4Cl etc., the so-called third negative wave of proteins (prenatrium wave [3-5]).

The mechanism of production of the double step wave of proteins has been studied by many investigators and especially by the polarographic school of Heyrovsky-Brdicka. It has been shown by the work of this school that in the process of occurrence of step II of the wave a great role is played by sulfhydryl groups, which catalyze the evolution of hydrogen on the mercury dropping electrode in an ammonia buffer [4, 6]:

$$-SH + \bar{e} \rightarrow -S^- + \frac{1}{2}H_2;$$

$$-S^- + H_2O \rightleftarrows -SH + OH^-;$$

$$NH_4^+ + OH \rightleftarrows NH_3 + H_2O.$$

However, Maironovskii [7] in the case of a polarographic investigation of sulfones suggested a somewhat different explanation of the mechanism of catalytic evolution of hydrogen:

$$2(R'SHR'')^+ + \bar{e} \rightarrow 2R'SR'' + H_2,$$

$$R'SR'' + H_2O \rightleftarrows R'SHR'' + OH^-;$$

where R'SR" is sulfide, R'SHR" is a sulfonium ion. In the case of protein R" designates two atoms of hydrogen.

We should also mention that there is evidence of participation of other groups in protein substances in the occurrence of step II waves [8]. The question of the nature of step I waves remains open. The mechanism of the occurrence of the single step protein has been little studied.

The wave of the proteinase of B. subtilis cannot be identified with step II wave or with the third negative wave of proteins, since there is no agreement with the half wave potentials.

In the present paper we raise the question of the conditions for the occurrence of the wave of the proteinase of B. subtilis and a comparison of the half wave potential of this enzyme with the half wave potential of other proteins.

METHODS

We studied 19 proteins and enzyme preparations. Among these were proteins which contained metallic ions (proteinase, bacterial amylase), porphyrins (catalase, hemoglobin), proteins rich in SH groups (papain, serum, and egg albumin). Some of the proteins were first treated polarographically (albumin and globulin of soy cotyledons). The purity of the preparations differed. Crystalline proteins: B. subtilis proteinase, catalase, pepsin, papain, α-amylase, takadiastase, serum albumin, γ-globulin, lysozyme, B. subtilis amylase. Highly purified preparations: Soy cotyledon albumin and globulin (electrophoretically homogeneous). Less pure preparations: Egg albumin edestin, sericin, hemoglobin, and extract from wheat bran. The preparations for analysis were obtained in water, 1 mg/ml, starting with an air dried sample. Moisture and enzyme activity were not considered, since our experiments had another purpose, study of the quality of the enzymes. The less soluble proteins were ground in a mortar before solution, and we used their filtrates in work.

Composition of the samples: 1 ml of protein (1 mg/ml) 1 ml of water and 3 ml of ammonia buffer (in the presence or absence of cobalt). Composition of the ammonia buffer: 2 g NH_4Cl and 2 ml of 17% ammonia in 100 ml of buffer mixture. To this we added $CoCl_2 \cdot 6H_2O$, 200 mg/100 ml of buffer. Variations from this composition of the sample are shown in the text. A description of the polarographic technique in this study was given earlier [2]. The time of dropping of the mercury (t) was 3.41 sec (at E = 1.286 v, first wave) and 3.1 sec (at E = 1.48 v, second wave, egg albumin). Height of mercury column 30 cm. Polarography carried out at 25° ± 0.5° without removing oxygen from the vessel. We used both photographic and visual recording in the work.

RESULTS

There is no indication in the literature of the mechanism of occurrence of a one step polarographic wave for proteins. The effect of hydrogen ion was studied in connection with the occurrence of a double step and prenatrium wave of protein and it was observed that, beginning at a definite pH value, the value for the sum of the heights of the double step waves remained constant. This fact was used as one of the indications of the catalytic nature of these waves [4-6, 10].

Along with the investigation of the effect on the height of the wave of B. subtilis proteinase, we also studied a protein which showed a double step wave, egg albumin.

For this purpose we used a buffer mixture with varying pH values: 5 ml of 2% NH_4Cl solution, 0.1-0.3 ml of 17% ammonia solution, 1 ml of a solution of $CoCl_2$ (120 mg/10 ml). Water was added to 10 ml and then we took 3 ml from it (for the experiment with egg albumin, 5 ml) and transferred it to a tube containing 1.5 ml of a solution of B. subtilis proteinase (0.5 mg/ml) or 4 ml of a solution of egg albumin (1 mg/ml) and 1-2 ml of a solution of agar-agar (1 mg/ml). Water was added to the sample to 10 ml. The results are given graphically in Fig. 1.

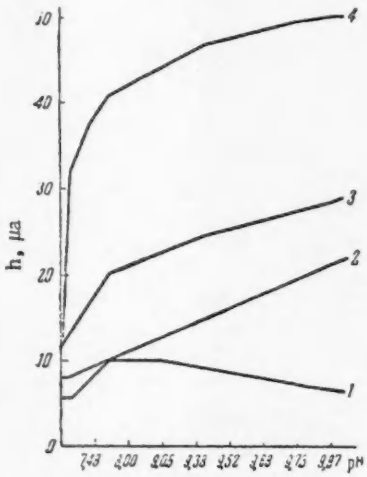

Fig. 1. Effect of pH on growth of proteinase waves. 1) Height of step I of egg albumin wave; 2) height of step II of egg albumin wave; 3) sum of egg albumin waves; 4) proteinase wave.

As Fig. 1 shows, the curves of relation of height of proteinase wave to pH differs from the curve of the sum of the catalytic waves of egg albumin. The height of the proteinase wave increases sharply at pH values 7.5-8.0, after which the rise in the curve becomes less. Evidently the pH of dissociation of electrically reduced groups in the proteinase and egg albumin differ, as the literature data confirm. In fact, the catalytic wave of egg albumin depends on SH groups while the occurrence of the proteinase wave depends on other groups, since there is no thioamino acid in the composition of this enzyme [9]. There are many diaminocarboxylic acids in the proteinase (hexone bases) which determine the alkaline isoelectric point of the enzyme (pH 10.5). It is possible that these amino acids take part in the reduction process at the dropping electrode.

In the next experiments we raised the question of the character of the processes which take part in the occurrence of the single step polarographic wave. In polarographic analysis we know the catalytic and adsorption-diffusion mechanisms of electrical reduction at the surface of the mercury drop. The catalytic process, as was mentioned above, is connected with the evolution of hydrogen on the dropping electrode, which, in turn, is connected with the buffer capacity (with increasing buffer buffer capacity at the same pH the catalytic evolution of hydrogen increases and produces an increased height of the wave).

To obtain buffer mixtures with different capacities we took a starting buffer mixture of 10 g NH_4Cl and 7.5 ml of 17% ammonia solution in 100 ml of mixture (C), after which it was diluted twice (B) and four times (A). To all the variants of the buffer we added 200 mg of $CoCl_2 \cdot 6H_2O$/100 ml of mixture. Then to the sample, which contained 2 ml (1 mg/ml) of solution of proteinase or egg albumin (in the experiments with albumin we used 1.5 ml of its solution), we added 1 ml (1 mg/ml) of starch solution, 5 ml of the corresponding buffer, and brought it to a total volume of 10 cm^3 with water. The sensitivity of the galvanometer was 1/200 (in the case of soy cotyledon albumin, 1/500). The results are given in Table 1.

We can conclude from the results in Table 1, that the single step wave of B. subtilis proteinase not only does not increase with increasing buffer capacity, but even drops somewhat. There is a particularly strong effect of increase in buffer capacity on the step I wave of egg albumin, in connection with which the ratio of the height of the wave (h) of step I to h of step II increases (Table 1, column C). This experiment gives no basis for considering the wave of the proteinase to be connected with catalytic evolution of hydrogen, as there is in the case of cotyledon albumin and egg albumin. We are left to assume that the occurrence of the proteinase wave is connected with the rate of diffusion of the protein molecules in the direction of the surface of the mercury drop. With increasing height of the mercury column the mass of the mercury flowing from the capillary increases, which produces an increase in the number of reduced protein molecules. The graphic relation between h and the square root of the height of the mercury column should have the form of a sloping straight line. In the catalytic process the straight line should be almost parallel to the abscissa. Figure 2 shows the increase in

TABLE 1. Effect of Capacity of Ammonia Buffer on Height of Protein Wave (height of wave given in μa)

Protein	Buffer mixture		
	A	B	C
B. subtilis proteinase	20,0	18,8	15,6
Cotyledon albumin (sum of waves)	18,4	21,6	40,8
Egg albumin (sum of waves)	27,6	37,6	106,0
Step I	11,2	18,6	66,0
Step II	16,4	19,2	40,0
Ratio of step I to step II	0,69	0,95	1,65

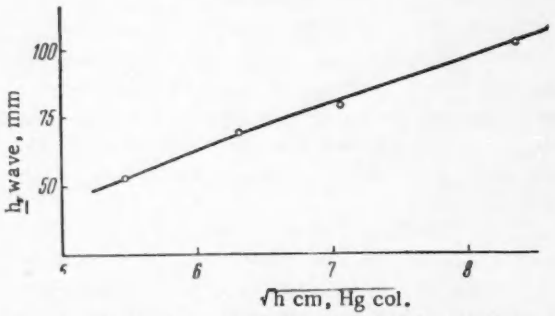

Fig. 2. Effect of increasing height of mercury column on height of proteinase wave.

height of the proteinase wave with increasing height of the mercury column, which indicates the diffusion character of the wave of this enzyme.

The solution of the problem of the position of the single step wave of the proteinase among the double step polarographic waves of the other proteins is closely connected with the determination and comparison of the half wave potentials of these substances. The question arises whether the proteinase wave is not identical with the step II wave of the double step waves of the other proteins. There is also the possibility of a chance agreement of E for the half wave of the other proteins, which might lead to an incorrect conclusion as to the character of the single step wave. Therefore, we carried out visual polarography with a series of proteins and enzymes to compare the half wave E of the two step waves in the Brdicka reagent, which contains divalent cobalt. In preliminary experiments we observed that the half wave E of proteins in the presence of cobalt is changed. This effect is closely connected with the shift in half wave E of cobalt ions to a more positive field of potential under the influence of the proteins. Sometimes the shift in half wave E reaches 100 mv.

In this connection, we ran experiments on the effect of different concentrations of papain on the position of half wave E of cobalt (Fig. 3). From the course of the curves in Fig. 3, we see the relation of half wave potential of divalent cobalt to concentration of papain. The shift in half wave E is completed when the protein con-

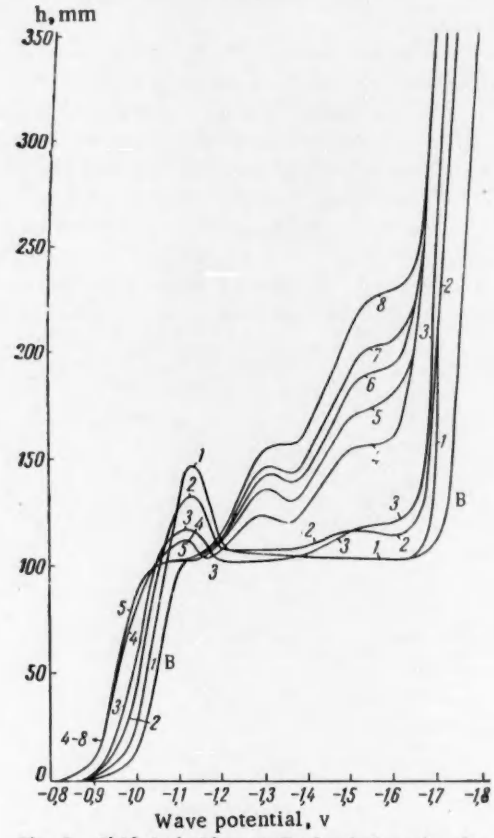

Fig. 3. Shift in half wave E of cobalt under the influence of papain. B – Base; 1) papain in concentration of 10 μ g/ml; 2) 30 μ g/ml; 3) 60 μ g/ml; 4) 80 μ g/ ml; 5) 100 μ g/ml; 6) 120 μ g/ml; 7) 140 μ g/ml; 8) 170 μ g/ml.

centration reaches about 100 μ g/ml. Further increase in papain concentration does not cause a shift of half wave E of cobalt. At a papain concentration 100 μ g/ml the maximum wave of cobalt disappears entirely and at 30 μ g/ml we observe a second step wave of the protein, and only at a papain concentration of 80 μ g/ml is there found the effect of the first step protein wave. We also observe a slight shift of step II half wave E of the protein (1.45-1.48 v) depending on the papain concentration.

Thus, within the limits of papain concentration 80-100 μ g/ml we find a close relation between the limits of shift of half wave E of cobalt, disappearance of the maximum wave of cobalt and appearance of the first step wave of protein. The disappearance of the polarographic maximum is fully explained on the basis of the Frumkin theory [11, 12].

Among the proteins which markedly shift the half wave E of cobalt are papain, lysozyme, serum albumin, cotyledon albumin, and egg albumin. All these proteins show a double step wave and have SH groups. However, though some proteins also show a double step wave they do not markedly shift the half wave E of cobalt to the more positive potential side (pepsin, sericin, bran extracts). The shift in half wave E of cobalt is explained by the formation of a complex compound of protein-cobalt. The nature of this complex is not clear, but it is clear that reduction of the divalent cobalt ion is activated, since the reduction process is completed at a more positive potential. Also, we must say that the valence of cobalt in the complex state is not changed, that is, cobalt is bound to the protein molecules by coordinate bonds.

In the next experiments we compared the half wave E of the proteinase with the half wave E of other proteins in the cobalt buffer solution of Brdicka. The proteins were studied within the limits of the concentration at which the half wave E of cobalt is not further shifted to the side of a positive potential. The results are given in Fig. 4.

Figure 4 shows that the half wave E of step I for serum albumin occurs at 1.26 v, for papain at 1.25 v, for pepsin at 1.24 v; that is, the half wave potentials of these proteins agree. Half wave E for step II of serum albumin occurs at 1.50 v, for papain at 1.475 v, for pepsin at 1.44 v; that is, the half wave potentials differ from each other by about 60 mv. The half wave E of proteinase occurs at 1.59 v and differs greatly from the half wave E of the above mentioned proteins (by about 130 mv compared to half wave E of step II wave and by 350 mv compared to the half wave E of step I for pepsin).

Comparative study showed that step I half wave E varies in the limits from 1.210 to 1.270 v and step II half wave E of the proteins from 1.410 to 1.505 v. For the third negative wave $E\frac{1}{2}$ of the proteins was studied on a base of ammonia buffer without cobalt. $E\frac{1}{2}$ for this wave was found in a voltage range of 1.620 to 1.780 v; proteinase on a base of ammonia buffer without cobalt showed a half wave potential in the more positive voltage field, at 1.550 v. Since the half wave of this enzyme on the base of the Brdicka reagent occurs at 1.590 v, then the single step wave of the proteinase cannot be considered the third negative wave of the proteins. The prenatrium wave of the proteinase cannot exist, since the basis for the existence of this wave is the reduction of the screened SH groups of the proteins [5]. Besides, it is known that the proteinase does not contain thioamino acids [9].

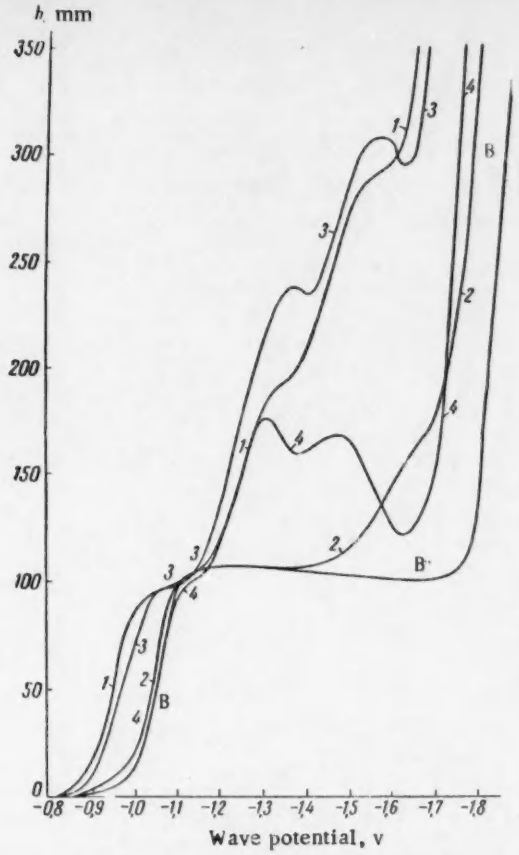

Fig. 4. Comparison of half wave E of proteinase with half wave E of some proteins. B – base; 1) papain; 2) proteinase; 3) serum albumin; 4) pepsin (final concentration in these proteins 200 μ g/ml).

However, on the base of ammonia buffer the proteinase shows a weak wave with half wave E at 1.71 v. The question of whether this can be considered a prenatrium wave remains open.

Thus, in polarographic behavior the proteinase differs considerably from the other proteins. This fact, along with the results reported in the literature [8], shows that in the production of the wave, groups other than SH, still little studied are involved.

Comparative data on half wave E of proteins, obtained on a base of ammonia buffer in the presence or absence of cobalt, are given in Table 2.

Globulin of soy cotyledons, edestin, α-casein, B. subtilis amylase, hemoglobin, and α-amylase have a prenatrium wave, although in cobalt ammonia buffer they are polarographically inactive. Some proteins at the extreme concentration of 200 μ g/ml show strong adsorption properties (Table 2) under the influence of which drops of uncharged mercury, passing through the polarographic solution which contains protein, are not combined into a single mass.

TABLE 2. Comparison of Half Wave E ($\frac{1}{2}$) of Some Proteins (against a saturated electrode)

Protein	Egg albumin	Serum albumin	γ-Globulin	Bean cotyledon albumin	Wheat bran extract	Sericin	Papain	Lysozyme	Pepsin	Takadiastase	Catalase	B. subtilis proteinase	Edestin	α-Casein	B. subtilis amylase	α-Amylase	Hemoglobin	Bean cotyledon glybulin
Base in the presence of Co																		
No. of wave steps	2	2	2	2	2	2	2	2	2	2	2	1						
$E_{1/2}$ Step I wave	−1,210	−1,245	−1,230	−1,235	−1,270	−1,250	−1,240	−1,260	−1,240	−1,220	−1,240	−						
$E_{1/2}$ Step II wave	−1,415	−1,505	−1,420	−1,450	−1,425	−1,350	−1,480	−1,45	−1,440	−1,425	−1,410	−1,590						
Base without Co																		
$E_{1/2}$ Wave absorption	−1,620 +	−1,190 +	−1,660 −	−1,160 +	− +	− +	− +	− +	−1,725 +	−1,660 +	−1,667 −	−1,550	−1,660	−1,680	−1,780 +	−1,680	−1,710	−1,650

The change in height of the wave of B. subtilis proteinase will permit us to study some of the denaturing processes of this enzyme in connection with its catalytic activity.

SUMMARY

The wave of B. subtilis proteinase has a diffusion character. Some proteins shift the half wave E of cobalt to a more positive reduction potential.

The limits of concentration at which the shift in half wave E stops agree with the occurrence of the step I wave of this enzyme. Proteinase does not show an effect on the shift of the half wave E of cobalt.

The half wave E of various proteins often differs. Half wave E of the single step wave of the proteinase occurs at 1.57-1.59 v and does not agree with the half wave E of the second step in the double step wave of other proteins (1.410-1.480 v) on a base of cobalt ammonia buffer.

The proteinase wave is also preserved in the absence of cobalt in the ammonia buffer (half wave E at 1.550 v) and does not correspond to the half wave potential of the third negative wave of the proteins. Along with the single step wave of the proteinase there is a weak wave with half wave E at 1.710 v. The question remains open as to the possibility of considering this to be a prenatrium wave.

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* Original Russian pagination. See C. B. translation.

THE DYNAMICS OF FORMATION OF CARBOHYDRATES IN THE VEGATIVE ORGANS OF HELIANTHUS TUBEROSUS L.

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Carbohydrates are the most widely distributed organic compounds of the plant kingdom, comprising up to 85-90% of the dry weight of the plant. The carbohydrates include a number of still insufficiently studied compounds, the so-called fructosans, which include inulin and a series of related substances which give fructose when hydrolyzed.

For a study of the dynamics of formation of carbohydrates, especially the fructosans, in plant organisms during ontogenesis we have chosen the Jerusalem artichoke Helianthus tuberosus L.

The Jerusalem artichoke is a valuable agricultural product; it gives 350-500 centners of green material and 200-250 centners of tubers per hectare. The green portion of the Jerusalem artichoke is used for preparing silage which is distinguished by its high nutritive quality. The relative richness of the Jerusalem artichoke fodder is 82%, the starch equivalant is 37.3, and the content of digestible protein is 6.1%[1]. The Jerusalem artichoke tubers are a valuable food for agricultural animals; in nutritive value they stand above the root vegetables.

Study of the transformations of carbohydrates in the vegetative organs of Helianthus tuberosus L. has been carried out be several authors. The study of Colin [2] showed that the sugars formed in the Jerusalem artichoke leaves, glucose, fructose, and sucrose, migrate from the leaves to the central part of the stem, where they are condensed into inulin. The condensation of the sugars to inulin occurs along the whole length of the stem, so that the amount of inulin increases in the lower part of the stem. Colin assumed that formation of inulin preceded the transformation starch ⇄ sucrose.

Schlubach and Florsheim [3] observed in Jerusalem artichoke leaves only reducing sugars and sucrose; they did not find fructosans in either the summer leaves or the autumn ones. Dedonde [4], using the chromatographic method, showed that the stems of the Jerusalem artichoke contained about ten different fructosans.

The vegetative organs of the Jerusalem artichoke contain sucrose; the question of the content of maltose in the leaves of this plant remains open. Other oligosaccharides are not found in the vegetative organs of the Jerusalem artichoke. The nonoccurrence of these substances is evidenly to be explained by the fact that in the quantitative determination of the alcohol soluble carbohydrates the easily hydrolyzed sugars were assumed to be sucrose without further study. However, at the present time there is evidence that the "sucrose fraction" of some plants contains, in addition to sucrose, easily hydrolyzed sugars which on hydrolysis form only glucose [5, 6].

The presence of starch in the Jerusalem artichoke leaves was established by Colin in France. Schlubach and Florsheim [3] analyzed the leaves of this plant in Germany and did not find starch in them. The absence of starch and maltose in the leaves of the Jerusalem artichoke of these investigators was explained by climatic peculiarities.

The studies of Lyubimenko [7] showed that the rate of deposition and solution of starch in the same plant or in different plants depended on the latitude in which the plants were grown. Lascombes and Carles [8] showed that the maltose content in the plants depended on the height above sea level of the location in which the plants were grown.

METHODS

The Jerusalem artichoke leaves which we analyzed contained starch and its hydrolysis products, dextrins.

For analysis of the carbohydrate complex of the Jerusalem artichoke vegetative organs we separated the carbohydrates by their solubility in alcohol, cold water, and hot water; starch was hydrolyzed by diastase, hemicellulose by 2% HCl; cellulose was first dissolved in 72% H_2SO_4 and then hydrolyzed by 3% H_2SO_4. The reducing sugars formed in the hydrolysis were determined by the ferricyanide method of Hanes [9].

RESULTS

Dynamics of the Accumulation of Carbohydrates in the Jerusalem Artichoke Leaves

For a study of the dynamics of formation of carbohydrates in Jerusalem artichoke leaves, these were collected for analysis monthly, at the beginning of each month (June to October) at 9 o'clock in the morning. Extraction and determination of the carbohydrates was carried out separately in the parenchyma of leaves and in the petioles along with the chief veins of the leaves. As the plants developed there was accumulation in the leaves of reducing sugars (Table 1) and fructose; at first glucose was formed and then fructose. It should be noted that fructose was first found in the petioles (in July) and

TABLE 1. Content of Reducing Sugars in Different Parts of the Leaves in the Period of Vegetation. (In this and later tables the sugar content is expressed in % of dry weight of the substance)

No. of leaves from below	Reducing sugars					Fructose included in this value			
	2.VI	3.VII	3.VIII	3.IX	3.X	3.VII	3.VIII	3.IX	3.X
Parenchyma of leaves									
51	—	—	—	2,11	6,29	—	—	1,10	4,79
44	—	—	—	1,77	5,32	—	—	0,97	4,66
37	—	—	—	2,35	6,61	—	—	1,35	5,85
30	—	—	1,70	2,01	4,51	—	—	1,05	4,28
23	—	—	1,96	2,06	—	—	—	1,31	—
16	—	1,43	1,46	1,42	—	—	—	0,98	—
8	0,28	1,86	2,10	—	—	—	—	—	—
4	0,23	—	—	—	—	—	—	—	—
Petioles and veins of leaves									
51	—	—	—	6,28	7,79	—	—	4,92	3,91
44	—	—	—	5,97	6,85	—	—	4,48	2,89
37	—	—	—	6,67	8,48	—	—	5,34	3,27
30	—	—	3,03	6,97	5,86	—	2,62	5,56	2,97
23	—	—	3,37	10,41	—	—	2,70	7,64	—
16	—	3,33	2,81	9,77	—	2,00	1,88	6,10	—
8	0,77	1,84	1,64	—	—	0,00	0,00	—	—
4	0,71	—	—	—	—	—	—	—	—

was found later in the parenchyma of the leaves (in Sept.).

In the quantitative determination of the oligosaccharides we were able to show an oligosaccharide of unknown nature in the vegetative organs of Jerusalem artichokes, which on hydrolysis gave glucose. This oligosaccharide will be conditionally called "biose". It should be noted that similar oligosaccharides are often observed in plants when there is more careful analysis of the carbohydrate complex. The results of the quantitative determination and differentiation of nonreducing sugars of Jerusalem artichoke leaves during the vegetative period of the plants are given in Table 2.

"Biose" was observed in the Jerusalem artichoke leaves earlier than sucrose; it was found in the leaves of young plants (at the beginning of June) and in the course of time, the amount of "biose" in Jerusalem artichoke leaves at first increased, and at the end of the vegetative period its content fell. Sucrose was observed in the leaves in the second half of the vegetative period (in September), and there was more of it in the petioles and veins than in the parenchyma of the leaves.

The determination of starch and dextrin content (in % of dry weight) in the leaf parenchyma at different parts of the vegetative period of the Jerusalem artichoke gave the following results:

These results show that the content of starch and dextrins in the Jerusalem artichoke leaves decreased with the age of the leaf.

The carbohydrates of the Jerusalem artichoke vegetative organs also include those soluble in hot water (pectin substances), hemicellulose, and cellulose. The data on the dynamics formation and of transformation of the polysaccharides in the Jerusalem artichoke leaf are given in Table 3.

Table 3 shows that the hot water soluble carbohydrates appear earlier in the leaf parenchyma than in the leaf parenchyma than in the petioles and veins.

Hemicelluloses are contained in relatively large amounts in young leaves; with development of the plant there occurs accumulation of these up to the time of formation of the tuber (to August), after which their content falls.

There is more cellulose than hemicellulose in young leaves. During development of the plant the content of both cellulose and hemicellulose falls in the leaf parenchyma and in the petioles and veins. It follows from this that the slightly mobile carbohydrates, which play a part as reserve substances, hemicellulose, and serve for formation of the supporting tissues, cellulose, also undergo transformations during vegetative growth of the plant.

No. of leaves from below	Starch					Dextrins				
	VI	VII	VIII	IX	X	VI	VII	VIII	IX	X
51	—	—	—	3,85	2,90	—	—	—	0,98	0,60
30	—	—	4,20	2,89	1,32	—	—	1,10	0,84	0,36
16	—	4,58	3,12	1,74	—	—	1,27	0,98	0,50	—
8	4,82	3,80	2,96	—	—	1,54	0,89	—	—	—
4	2,64	—	—	—	—	0,78	—	—	—	—

TABLE 2. Content of Nonreducing Sugars in Jerusalem Artichoke Leaves during the Vegetative Period

No. of leaves from below	"biose"					Sucrose	
	VI	VII	VIII	IX	X	IX	X
Parenchyma of leaves							
51	—	—	—	2,53	0,65	0,51	3,61
44	—	—	—	1,52	0,77	0,50	3,00
37	—	—	—	2,08	0,99	0,61	4,69
30	—	—	0,54	0,73	1,53	0,81	2,01
23	—	—	1,19	1,23	—	0,51	—
16	—	1,24	0,98	0,93	—	0,31	—
8	0,21	1,09	0,43	—	—	—	—
4	0,37	—	—	—	—	—	—
Petioles and Veins of leaves							
51	—	—	—	1,76	1,86	2,29	5,19
44	—	—	—	2,65	4,69	3,02	5,34
37	—	—	—	0,33	2,33	4,09	7,95
30	—	—	1,68	0,76	2,77	3,13	3,23
23	—	—	1,84	1,54	—	2,72	—
16	—	1,64	0,89	2,46	—	2,14	—
8	0,60	0,82	1,00	—	—	—	—
4	0,41	—	—	—	—	—	—

TABLE 3. Content of Polysaccharides in the Jerusalem Artichoke Leaf in the Period of Vegetation

No. of leaves from below	Carbohydrates soluble in hot water			Hemicellulose					Cellulose				
	VIII	IX	X	VI	VII	VIII	IX	X	VI	VII	VIII	IX	X
Parenchyma of leaves													
51	—	1,39	3,00	—	—	—	2,87	2,07	—	—	—	13,34	9,17
44	—	0,64	0,81	—	—	—	2,46	1,08	—	—	—	13,52	10,66
37	—	0,76	0,93	—	—	—	2,39	1,27	—	—	—	12,82	10,01
30	1,54	0,86	0,91	—	—	3,00	2,24	1,06	—	—	12,34	17,21	14,06
23	0,76	0,66	—	—	—	3,53	2,30	—	—	—	15,80	16,06	—
16	1,31	0,93	—	—	1,35	4,46	1,97	—	—	14,06	14,03	16,56	—
8	0,94	—	—	2,96	1,63	3,18	—	—	16,15	14,51	15,53	—	—
4	—	—	—	3,08	—	—	—	—	17,56	—	—	—	—
Petioles and veins													
51		0,87	2,99	—	—	—	3,91	3,53	—	—	—	16,25	13,51
44		0,88	3,14	—	—	—	3,90	4,24	—	—	—	14,82	10,39
37		1,05	3,75	—	—	—	4,31	2,99	—	—	—	16,48	12,54
30		0,76	2,25	—	—	6,67	3,91	2,25	—	—	16,31	15,69	15,57
23		1,07	—	—	—	5,70	3,80	—	—	—	17,76	18,48	—
16		0,92	—	—	4,53	4,98	3,38	—	—	20,65	18,79	24,29	—
8		—	—	4,76	5,24	7,07	—	—	18,18	22,28	20,88	—	—
4		—	—	4,97	—	—	—	—	19,09	—	—	—	—

The anatomical picture of delignification in annual plants has been described by several investigators who concluded that the lignification process is reversible. Aleksandrov [10] showed that spiral, annular, and, perhaps, sieve like vessels almost always were delignified and absorbed. The process of breakdown of cell walls was found by Paleev [11] in the analysis of rye; on the basis of his studies this author concluded that the components of the cell walls of leaves and stems after their delignification served as sources for building the rye grain.

Dynamics of Accumulation of Carbohydrates in Jerusalem Artichoke Stems

In the study of the dymanics of accumulation of carbohydrates in the stems, the latter were divided into a definite number of levels corresponding to the number of analyzed leaves in such a way that the middle portion of the corresponding level of the stem was compared with the analyzed leaf. Each portion (level) of the stem was first divided into two parts: the bark and the wood along with the pith (spongy mass); when formation of the pith occurred along the whole length of the stem, the latter was separated into three parts: bark, wood, and pith. The results of the determination of reducing sugars in different parts of the stem during development are given in Tables 4 and 8.

Table 4 shows that with development of the plant there occurs an accumulation of reducing sugars whose maximum content is found in the pith of the lower levels of the stem at the end of the period of vegetation

TABLE 4. Content of Monosaccharides in Different Parts of the Stem during the Vegetative Period

Stem level from below	Reducing sugars					Fructose included in these				
	VI	VII	VIII	IX	X	VI	VII	VIII	IX	X
Stem bark										
7	—	—	—	6,83	6,02	—	—	—	3,95	3,05
6	—	—	—	6,54	5,76	—	—	—	3,68	2,92
5	—	—	2,57	8,95	6,90	—	—	0,77	4,83	3,19
4	—	—	3,01	8,57	6,17	—	—	2,23	4,44	3,10
3	2,31	2,98	4,15	11,24	6,68	—	—	3,18	6,09	4,36
2	1,32	4,55	5,70	10,53	5,69	—	3,02	3,76	8,16	3,14
1	2,07	8,41	8,14	9,40	4,95	1,29	4,19	3,35	8,08	3,33
Stem wood										
7	—	—	—	11,82	6,26	—	—	—	6,64	2,98
6	—	—	—	11,57	6,82	—	—	—	8,43	2,72
5	—	—	11,53	10,16	6,26	—	—	—	7,94	2,90
4	—	—	12,29	10,35	6,48	—	—	8,06	7,29	2,97
3	5,40	9,41	10,59	10,07	6,33	—	—	5,74	6,05	2,80
2	4,45	5,93	9,06	8,88	6,54	—	2,87	5,14	4,22	2,99
1	5,09	2,41	8,74	4,17	3,85	4,57	2,03	3,55	1,74	1,90

TABLE 5. Content of Nonreducing Sugars in Jerusalem Artichoke Stems at the End of Vegetation

Stem level from below	September 3				October 3			
	Total nonreducing sugars	included in this			Total nonreducing sugars	Included in this		
		"biose"	sucrose	fructose		"biose"	sucrose	fructose
Stem bark								
7	5,80	0,56	5,24	—	19,78	2,48	9,60	7,70
6	6,88	0,88	3,54	2,46	18,77	4,59	6,89	7,29
5	8,79	1,98	3,51	3,30	17,48	5,60	4,62	7,26
4	11,05	2,65	4,76	3,64	21,38	6,84	6,84	7,70
3	9,17	2,51	2,70	3,96	18,69	5,31	5,81	7,57
2	10,86	2,97	3,05	4,84	24,03	6,26	5,26	12,51
1	9,79	2,47	1,77	5,55	25,85	3,92	4,28	17,65
Stem wood								
7	23,30	3,01	14,24	6,05	25,82	—	6,04	19,78
6	24,56	6,00	12,45	6,11	24,76	—	7,90	16,86
5	21,49	5,87	9,55	6,07	22,17	1,34	7,00	13,83
4	20,67	7,55	6,71	6,41	22,08	3,12	6,01	12,95
3	18,29	8,52	5,02	4,75	22,16	6,67	3,51	11,98
2	16,81	9,00	3,66	4,15	18,91	5,75	2,23	10,93
1	12,31	7,83	2,60	2,87	13,83	6,50	1,66	5,67
Stem pith								
7	23,92	7,56	15,61	0,75	14,64	3,52	9,89	1,23
6	22,41	5,80	14,37	2,24	13,30	1,13	10,07	2,10
5	23,29	6,29	13,13	3,87	14,49	2,56	11,32	3,17
4	28,48	5,64	16,09	6,75	25,30	2,26	14,86	8,18
3	28,20	3,84	14,57	9,79	30,37	2,05	18,02	10,30
2	25,84	2,58	12,61	10,55	34,44	1,37	20,76	12,31
1	23,30	1,86	8,44	13,00	35,61	1,13	24,63	9,85

(Table 8). Free fructose appears in the axial part of the plant earlier (June) than in the leaf petioles, and the lower the level of the stem, the earlier it appears. The data on the content of nonreducing sugars in various parts of the Jerusalem artichoke stem at the end of the vegetative period, September-October, appear in Table 5.

In the stem bark, the "biose" content increases from the upper levels to the lower, while the sucrose

TABLE 6. Content of Carbohydrates Soluble in Hot Water in Jerusalem Artichoke Stems

Stem level from below	June	July	August	September	October
			Stem bark		
7	—	—	—	1,18	2,02
6	—	—	—	2,02	4,97
5	—	—	0,39	2,45	5,70
4	—	—	1,63	2,83	3,70
3	0,0	1,81	1,29	2,69	2,43
2	0,0	1,53	0,94	2,28	1,78
1	0,0	1,00	1,14	2,85	4,59
			Stem wood		
7	—	—	—	11,30	2,70
6	—	—	—	16,50	4,70
5	—	—	,37	17,30	4,60
4	—	—	2,96	12,80	2,30
3	0,15	0,94	1,79	9,00	1,40
2	0,88	1,58	4,05	10,20	2,00
1	5,72	1,02	4,72	10,70	2,50

TABLE 7. Content of Polysaccharides in Different Parts of the Stem of the Jerusalem Artichoke by Months

Stem level from below	Hemicellulose					Cellulose				
	VI	VII	VIII	IX	X	VI	VII	VIII	IX	X
					Stem bark					
7	—	—	—	5,71	5,84	—	—	—	18,21	19,20
6	—	—	—	4,79	4,19	—	—	—	22,28	25,45
5	—	—	7,86	4,91	5,29	—	—	25,78	21,73	23,55
4	—	—	8,94	6,04	5,70	—	—	25,30	24,51	26,21
3	5,20	6,42	6,35	5,36	4,28	17,80	18,69	25,48	20,83	22,03
2	3,42	6,01	6,53	4,59	3,57	18,59	20,12	25,04	19,54	22,52
1	4,49	7,28	7,43	5,10	3,64	21,24	21,78	24,08	16,62	18,19
					Stem wood*					
7	—	—	—	2,79	1,99	—	—	—	13,06	15,86
6	—	—	—	2,94	1,97	—	—	—	15,02	24,66
5	—	—	4,71	3,26	1,92	—	—	16,95	13,55	23,33
4	—	—	4,66	2,57	2,04	—	—	16,97	13,56	22,81
3	2,99	4,73	5,36	2,94	1,09	29,34	25,89	18,83	15,17	25,30
2	2,82	5,14	4,17	3,49	2,84	22,56	21,93	22,05	18,78	27,40
1	3,82	8,59	3,53	2,66	1,55	25,91	28,25	25,34	24,52	31,22

*In July-August the stem was analyzed along with the pith.

TABLE 8. Carbohydrate Content in the Jerusalem Artichoke Stem Pith in September-October

Stem level from below	Reducing sugars		Fructose		Carbohydrates soluble in hot water		Hemicellulose		Cellulose	
	IX	X	IX	X	IX	X	IX	X	IX	X
7	16,96	7,40	5,78	6,04	12,60	1,10	3,65	6,39	16,01	24,12
6	15,06	9,15	7,10	6,96	12,20	1,10	4,32	6,24	16,44	26,80
5	19,89	11,73	10,99	11,08	15,80	1,20	3,81	5,32	16,80	28,77
4	23,05	16,63	13,47	14,42	10,00	2,00	4,08	5,41	15,62	26,17
3	26,37	18,74	14,12	15,96	8,70	2,50	2,01	2,98	12,51	24,84
2	27,98	22,93	17,55	21,12	6,60	6,55	2,17	2,25	12,94	18,55
1	27,59	23,90	18,84	22,58	7,90	8,20	1,75	2,33	14,67	18,65

content decreases in the same direction. During this period of plant development there also appear in the bark the alcohol soluble fructosans whose content increases from the upper to the lower levels of the stem. In the stem wood there is a decrease in content of nonreducing sugars from the upper levels to the base of the stem. There is more "biose" in the wood than in the stem and its content increases toward the bottom, that is, the older part of the stem. Evidently "biose", as as earlier formed sugar, plays the part of an intermediate compound which is transformed into other kinds of sugars, and is probably also a precursor of cellulose, since wherever there is more cellulose (stem wood) there is also more "biose". This is the more likely, since in recent times the anhydride of glucose, cellobiose, has been found in plants [12].

The alcohol soluble fructosans are present in greater amount in the wood than in the bark, and their amount decreases from the upper to the lower levels of the stem. In the pith portion of the stem there is an accumulation of total amount of reducing sugars from the upper levels to the base of the stem (this is found especially in October); the "biose" content falls from the upper to the lower levels of the stem, and the content of sucrose increases in the same direction. The content of alcohol soluble fructosans in the stem pith, like the total amount of nonreducing sugars, increases toward the base of the stem.

Determination of the content of water soluble (insoluble in 90% alcohol) fructosans in % of dry weight is as follows:

Stem level from below	wood		pith	
	September	October	September	October
7	2,36	10,95	2,33	10,31
6	4,31	12,22	2,53	11,73
5	5,54	10,56	2,73	9,60
4	5,02	9,81	3,17	7,44
3	4,79	7,97	2,67	5,00
2	6,69	6,50	2,10	2,45
1	5,81	4,39	1,63	2,39

Thus, the water soluble fructosans in the Jerusalem artichoke stems are less than the alcohol soluble fructosans, and their content at the end of the vegetative period decreases from the upper to the lower levels of the stem.

The content of hot water soluble carbohydrates (pectin substances) in the Jerusalem artichoke stems (Tables 6 and 8) increases with the development of the plant, reaching a maximum in the wood and pith in September; later their content falls. In the bark of the stem accumulation of these carbohydrates occurs at a later time, in October.

The data of Tables 7 and 8 show that during development of the plants the hemicellulose content in the bark and wood of the stems decreases and the content of cellulose rises.

The carbohydrates formed in the vegetative organs of the Jerusalem artichoke migrate to the tubers through the stolons, the fructosan complexes of which change as follows during the vegetative period:

Carbohydrates (in % dry weight)	July	August	September	October
Fructose	9,34	7,66	4,54	1,59
Sucrose	3,46	6,08	7,43	9,89
Fructosans soluble in 90% alsohol	12,85	8,96	6,80	3,08
Fructosans insoluble in 90% alcohol	5,04	13,26	38,70	46,12

These results show that in the stolon there occurs a process of polymerization of low molecular weight fructosans (soluble in alcohol) into more complex fructosans (insoluble in alcohol) whose amount steadily rises due to decrease in amount of the first.

SUMMARY

In the Jerusalem artichoke leaves glucose is first formed; fructose appears later than glucose and the first fructose is found in the pitioles and veins, and then in the leaf parenchyma. In the axial part of the plant fructose appears earlier than in the leaves, and in the lowest levels of the stem it appears earliest.

Among the oligosaccharides an anhydride of glucose, "biose", is first formed and is evidently a precursor from which are formed the polysaccharides of the cell membranes. Sucrose appears in the plants after "biose" but earlier than the alcohol soluble fructosans and it is possible that sucrose is the substance from which the alcohol soluble fructosans are formed.

Low molecular weight fructosans are formed chiefly in the bark of the stem and their amount increases from the upper to the lower levels of the stem. The more complex fructosans are preferentially found in the wood and pith of the stem which shows that the polymerization of low molecular weight fructosans into high molecular weight ones occurs when the first migrate from the periphery to the center of the stem.

During the development of the plant there is a decrease in content of hemicellulose and cellulose in various parts of the leaf and in some levels of the Jerusalem artichoke stem. The results of investigation of the chemical composition show that at the end of the period of vegetation the Jerusalem artichoke stem, especially its lower part, can be used as a source of carbohydrates.

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THE EFFECT OF SOME HALOGEN DERIVATIVES OF PHENYLALANINE ON THE DECARBOXYLASES OF STREPTOCOCCUS FAECALIS

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A considerable number of studies have appeared in recent years devoted to the investigation of the effect on enzymes of various substances which are structurally close to the substrates. The data which have been obtained permit the suggestion of the nature of the chemical bonds which take part in the formation of the enzyme-substrate complex, and also allow some conclusions to be drawn as to the structure of the active centers of the enzymes.

In seeking possible inhibitors of amino acid decarboxylases, it has been found that the tissue decarboxylase of "dopa" (3,4-dihydroxyphenylalanine) is inhibited by various compounds: a number of amines [1-3], some folic acid antagonists [4], derivatives of cinnamic acid and chalcones [5], and methylated derivatives of phenylalanine [6]. The last two groups of compounds to a lesser, but considerable, extent inhibit also the decarboxylation of tyrosine by the corresponding bacterial decarboxylases. From the point of view of a study of the requirements in the structure of the substrate for enzymatic catalysis, the data given by different analogs of specific substrates are of the greatest value.

In our experiments we have studied the action on the decarboxylase of Streptococcus faecalis of some phenylalanine derivatives obtained by substituting halogens for one or two hydrogen atoms in the phenylalanine nucleus. The interest in such compounds is due to the known fact that introduction of halogens in the aromatic ring results in formation of physiologically active compounds. For example, dichlorophenoxyacetic acid is a powerful synthetic phytohormone [7], 2,4-dichlorophenylalanine (2,4-DCPA) retards the growth of Leuconostoc mesenteroides [8] and Saccharomyces cerevisiae [9], various fluoro- and chloroderivatives of phenylalanine inhibit growth and adaptive formation of maltase in yeasts [10].

EXPERIMENTAL

Cultures of Streptococcus faecalis, strains 6783, 6782, and 6459 (English National Collection of Cultures, London), were grown on meat-peptone medium, pH 6.8, for 24 hours at 37° [11]. The source of the decarboxylase was either a dry acetone preparation (15 mg per sample) or a suspension of live Streptococcus faecalis (calculated at 15 mg of dry cells in the sample). Determination of the dry weight of the cells in the suspension was carried out by measuring the cloudiness in a Hilger absorptiometer with later calculation of the dry weight from a calibration curve set up for this microorganism. In making the acetone preparation, the bacterial mass, twice washed with distilled water, was precipitated by acetone, washed with a mixture of acetone and ether (1:1) and with ether, and air dried at room temperature. The entire operation was carried out in the cold. Decarboxylase activity was determined by a manometric method in a Warburg apparatus at 37° in an air atmosphere.

The reaction mixture (3 ml) in the central part of the vessel contained the studied substance (antimetabolite), the acetone preparation or the live bacterial cells, and 0.02 M acetate buffer, pH 5.6. In the side arm was placed the substrate for decarboxylation. The phenyl alanine analogs (antimetabolites) were incubated with the enzyme for 20 min before adding the substrate.

The amino acids used in the experiments were L-isomers, the antimetabolites were racemates: LD-2,4-DCPA, LD-p-chlorophenyl alanine (p-CPA), and LD-p-bromophenyl alanine (p-BPA).*

The effect of some phenylalanine analogs on "dopa"-decarboxylase can be judged from the data given in Table 1.

All the substances which we studied inhibited the action of decarboxylase, and the inhibiting effect was shown considerably more strongly in the experiments with live microorganisms. We observed that p-BPA, which was less active than p-CPA in the experiments with the acetone preparations was more active, when suspensions of live cells were used, than was the corresponding chloroderivative. Also, inhibition of decarboxylase was stronger in the experiments with live cells than with acetone preparations. The reason for this is not clear.

The strongest inhibitor in both cases was 2,4-DCPA. In the presence of 0.82 mg of "dopa" and 1.95 mg of 2,4-DCPA, decarboxylation of the amino acid by live bacterial cells was practically 100% inhibited (Table 1). The index of inhibition, that is, the ratio of concentrations of the two substances at which their action is

* The analogs were synthesized by A. M. Yurkevich [12] and kindly presented to us, for which we express our thanks.

TABLE 1. Inhibition of "Dopa"-Decarboxylase (%) in the Presence of Phenyl Alanine Derivatives (Concentration: "dopa" – $1.3 \cdot 10^{-3}$ M; 2,4-DCPA – $2.8 \cdot 10^{-3}$ M; p-CPA – $3.0 \cdot 10^{-3}$ M; p-BPA – $2.8 \cdot 10^{-3}$ M

Incubation, min	Acetone preparation			Live cell suspension		
	2,4-DCPA	p-CPA	p-BPA	2,4-DCPA	p-CPA	p-BPA
5	58,1	23,1	14,9	96,2	33,8	51,2
10	53,5	15,0	11,2	92,2	25,6	40,4
15	45,6	10,1	8,7	80,3	15,6	30,6
20	37,6	8,6	5,0	77,6	16,0	25,2
25	27,8	4,2	0	66,3	14,5	21,1
30	25,3	0	0	52,0	10,8	17,7

TABLE 2. Inhibition of "Dopa" Decarboxylase (%) with Change in Concentration of Substrate and Analog (ratio 1 : 2) (acetone preparation of Streptococcus faecalis used)

Incubation, min	"Dopa" $2.8 \cdot 10^{-3}$ M; 2,4-DCPA $5.6 \cdot 10^{-3}$ M	"Dopa" $1.4 \cdot 10^{-3}$ M; 2,4-DCPA $2.8 \cdot 10^{-3}$ M	"Dopa" $0.7 \cdot 10^{-3}$ M; 2,4-DCPA $1.4 \cdot 10^{-3}$ M
5	67.0	73.8	74.6
10	68.0	67.8	75.4
15	70.7	76.8	69.3
20	70.9	66.5	60.0
25	70.0	62.5	51.1
30	64.4	54.5	44.0

equalized was 2.4 (that is, for complete inhibition of the decarboxylase there must be 2.4 times more 2,4-DCPA than "dopa"). Comparison with the literature values on index of inhibition for different systems [13] shows that this analog has a comparatively high inhibiting activity.

Starting from present ideas about the nature of antagonism among closely related structures, we can assume that the structural similarity of analogs to the substrate assures their combination with a specific protein. From this point of view the closest analog to "dopa" would obviously be 3,4-DCPA. Among the phenyl-alanine derivatives available to us 2,4-DCPA has a greater structural similarity to the substrate than p-CPA or p-BPA, which is shown by the stronger inhibition of "dopa" decarboxylase by this compound.

If the reaction of formation of the enzyme-inhibitor complex is reversable, then it competes with the reaction of formation of the enzyme-substrate complex, and the preference for one or the other reaction depends on the relative concentrations of the competing compounds. If the ratio of concentration of substrate and of analog needed to give a definite effect have the same values over a wide range of concentrations, then, as is known, such inhibition is called competitive.

The data for the character of the inhibition in the case of the action of 2,4-DCPA on "dopa" decarboxylase are given in Table 2.

Starting from these data we can assume that we are dealing with a competitive or nearly competitive inhibition. However, such a conclusion is highly relative, first because we cannot study the action of 2,4-DCPA over a wider range of concentrations (5, 10, 100 times more or less) because of the definite limitations of the manometric method which we used, and the comparatively poor solubility of "dopa" and 2,4-DCPA.

Stricter evidence for the character of the inhibition was obtained by mathematical treatment of the data, which we could express graphically by the method of double inverse values [14]. In this case, for competive inhibition the dependence of $1/v$ (v is reaction rate) on $1/s$ (s is concentration of substrate) is given by a straight line on condition that the inhibitor concentration remains constant with change in concentration of the substrate. These straight lines intersect at a point which lies on the ordinate. Below, in Table 3 and in the figure, we give the treatment thus obtained from the data.

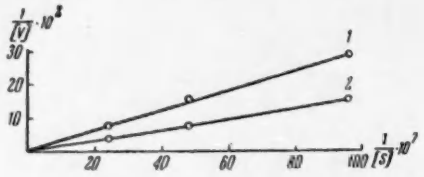
Using a different "dopa" concentration, we obtained the same graphic expression of dependence, that is, evidence for competitive inhibition. The presence of a competitive inhibition in this case also emphasizes the fact that the effect from the action of 2,4-DCPA decreases with increasing "dopa" concentration (Table 3).

It is known that phosphopyridoxal reacts with different amines and amino acids [15] including "dopa", so that it can be suggested that the inhibiting effect of 2,4-DCPA on "dopa" decarboxylase depends on a compound analogous to a coenzyme. In our experiments, the addition of phosphopyridoxal (15 μg/sample) as would be expected, activated the decarboxylation of "dopa", but removing or decreasing the inhibition process in the presence of 2,4-DCPA was not observed. Thus, inhibition of the enzyme in this case does not depend on reaction of 2,4-DCPA with the coenzyme.

Starting from the facts described in the literature that some substances have a diphasic action, inhibiting processes at high concentrations and activating than at low concentrations [13, 16], we studied the effect of different concentrations of 2,4-DCPA on "dopa" decarboxylase. As Table 4 shows, decreasing the concentration of 2,4-DCPA 10 times compared to the concentration which

produces half the maximum inhibition produces full disappearance of the inhibiting effect, but activation of the enzyme does not occur, even with as much as 10,000 times decrease in the concentration of 2,4-DCPA.

During the work, it was shown that halogen derivatives of phenylalanine also inhibit microbial tyrosine decarboxylase (Streptococcus faecalis, Table 5), and in



Effect of 2,4-DCPA on "dopa" decarboxylase. 1) Sample with inhibitor; 2) without inhibitor. Competitive inhibition shown by the method of double inverse values (Table 3).

TABLE 3. Effect of 2,4-DCPA on Rate of Reaction (microliters CO_2/min) of "Dopa" Decarboxylation (We give average values from data obtained for 6, 9, and 12 min. 2,4-DCPA concentration $2.8 \cdot 10^{-3}$ M; acetone preparation of Streptococcus faecalis)

Substrate concentration, M	"Dopa"	"Dopa" + 2,4-DCPA
$4.2 \cdot 10^{-3}$	19,9	12,2
$2.1 \cdot 10^{-3}$	12,2	7,2
$1.05 \cdot 10^{-3}$	7,1	3,7

experiments with suspensions of living bacterial cells inhibition is about the same for all the compounds which we studied. This evidently follows the rule, if we consider that substitution of a hydrogen atom in the para position by a halogen at once creates an analog structurally closer to tyrosine (p-CPA, p-BPA). 2,4-DCPA can be regarded as the same analog with a supplementary substitution of hydrogen by halogen in the ortho-position. Somewhat unexpected in this aspect is the absence of inhibition by p-CPA in work with the actone preparation. At the present time we do not have enough data to explain this fact.

Besides the mentioned decarboxylases of Streptococcus faecalis, we have studied the action of halogenoaryl alanines on the phenyl alanine decarboxylase of this organism.

In studying Streptococcus faecalis we found that strains 6459 and 6782, besides the ability to decarboxylate tyrosine and "dopa" [17, 18] also had a somewhat weaker phenylalanine decarboxylase activity. The enzyme did not form in a growing culture of strain 6783 on a meat peptone medium even with addition of considerable amounts of phenylalanine. McGilvery and Cohen [19] also reported the presence of a phenylalanine decarboxylase in this organism with an activity of the same order as that noted by us. The final product of the decarboxylation, phenylethylamine, was isolated by the authors and identified as the corresponding benzoic acid amide and benzenesulfonic acid amide.

The effect of halogen substituted analogs of phenylalanine on the phenylalanine decarboxylase of Streptococcus faecalis (strain 6459) is shown by the data given in Table 6.

It is interesting to see that, differing from the inhibiting action of 2,4-DCPA on "dopa" and tyrosine decarboxylases, in this case 2,4-DCPA is less effective.

TABLE 4. Inhibition of "Dopa" Decarboxylation (%) in the Presence of Different Amounts of 2,4-DCPA ("Dopa" concentration – $1.3 \cdot 10^{-3}$ M; acetone preparation of Streptococcus faecalis)

Incubation, min	2,4-DCPA concentration, M					
	$2.8 \cdot 10^{-3}$	$5.6 \cdot 10^{-4}$	$2.8 \cdot 10^{-4}$	$2.8 \cdot 10^{-5}$	$2.8 \cdot 10^{-6}$	$2.8 \cdot 10^{-7}$
5	58,0	18,2	0	0	0	0
10	55,0	15,0	0	0	0	0
15	43,3	8,2	0	0	0	0
20	37,1	1,2	0	0	0	0

TABLE 5. Inhibition of Tyrosine Decarboxylation (%) in the Presence of Halogen Derivative of Phenyl Alanine (Concentrations: Tyrosine – $1.4 \cdot 10^{-3}$ M; 2,4-DCPA – $2.8 \cdot 10^{-3}$ M; p-CPA – $3.0 \cdot 10^{-3}$ M; p-BPA – $2.8 \cdot 10^{-3}$ M

Incubation, min	Acetone preparation			Suspension of live cells		
	2,4-DCPA	p-CPA	p-BPA	2,4-DCPA	p-CPA	p-BPA
5	51,5	6,0	24,7	79,3	58,0	56,8
10	18,5	0	12,3	52,9	50,0	48,7
15	3,0	0	4,0	43,1	27,0	26,5
20	0	0	0	27,2	23,0	24.0

TABLE 6. Inhibition of Phenylalanine Decarboxylation in the Presence of Phenylalanine Derivatives (Concentrations: Phenylalanine - $1{,}4 \cdot 10^{-3}$ M; 2,4-DCPA - $2{,}8 \cdot 10^{-3}$ M; p-CPA - $3.0 \cdot 10^{-3}$ M; p-BPA — $2.8 \cdot 10^{-3}$ M; acetone preparation of Streptococcus faecalis

Incubation, min	2,4-DCPA	p-CPA	p-BPA
5	30,0	69,3	71,7
10	27,1	56,0	69,0
15	26,0	52,0	62,2
20	26,0	40,7	49,7

SUMMARY

We have established that 2,4-dichlorophenyl alanine, p-chloro-, and p-bromophenyl alanine inhibit the "dopa" decarboxylase of Streptococcus faecalis. 2,4-DCPA is the most active inhibitor. Inhibition of "dopa" decarboxylase by 2,4-dichlorophenyl alanine is of a competitive character. The analog does not have a diphasic action, that is, it does not activate the enzyme at low concentrations ($2{,}8 \cdot 10^{-4}$ to $2{,}8 \cdot 10^{-7}$ M).

The tyrosine decarboxylase and phenylalanine decarboxylase of Streptococcus faecalis also are inhibited by halogen substituted aryl alanines; for phenylalanine decarboxylase we observed a weaker inhibiting action for 2,4-dichlorophenyl alanine as compared to p-chloro- and p-bromophenyl alanine.

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THE PREPARATION OF PURE PHOSPHORUS ESTERS OF THIAMINE-S^{35}

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In recent years vitaminologists in various countries have sought more active forms of vitamins, and especially of coenzymes. Considerable success has been attained in getting cocarboxylases. The coenzyme function of cocarboxylase (thiamine pyrophosphate, TPP) was discovered in 1911 [1]. Since that time there have been many studies of the catalytic properties of TPP [2]. At the present time there are studies of the catalytic properties of thiamine triphosphate (TTP) and thiamine monophosphate (TMP) which in the animal organism can be transformed more rapidly into cocarboxylase than can thiamine.

The present work is devoted to developing a method for obtaining pure solutions of TMP, TPP, and TTP, labeled on the sulfur from small amounts of starting thiamine-S^{35} (5-40 mg).

EXPERIMENTAL

Obtaining a Mixture of Phosphorus Esters of Thiamine-S^{35}

At first (1933) TPP was obtained from yeast [3-5], when its structure was established [5], and TMP was obtained from TPP by acid hydrolysis. Later (1937) the chemical synthesis of TPP was carried out by phosphorylation of thiamine [6-9], and in the precipitation and purification of the resulting coenzyme the same methods were used as had been applied for separation of TPP from yeast (precipitation by tungstate, acetone, and adsorption). The most successful chemical synthesis of TPP was suggested by Weijlard [7, 8]. His method was perfected by Karrer and Viscontini [10]. This modification of the TPP synthesis has been used until the present time [11], in spite of the difficulty and length of purification of TPP from the accompanying thiamine phosphorus esters. Following a suggestion of Velluz [12], Viscontini, Bonetti, and Karrer [13], worked out a simple method for phosphorylation of thiamine directly to TTP with later acid hydrolysis to TPP. Both phosphorus esters of thiamine were fractionated by precipitation with acetone. Various modifications of this basic method were developed and more completely generalized in the detailed work of Serchi [14]. In this work, the author, using chromatographic analysis of different TPP preparations, showed their nonhomogeneity (in particular, commercial preparations contained from 76-88% TPP). The reality of this conclusion was confirmed in numerous studies [15, 16], the authors of which studied the homogeneity of TTP and TPP obtained according to Viscontini [13] by chromatography and electrophoresis, and showed their nonhomogeneity.

In a study of the possibility of using the above methods for obtaining labeled TTP, TPP, and TMP, we selected the method suggested by Viscontini and coworkers [13] for the synthesis of TTP. That which many other investigators [15, 16], had considered a weakness of the method (formation in the phosphorylation of thiamine of considerable quantities of other phosphorus esters of thiamine besides TTP) was actually very suitable for the simultaneous preparation from one portion of thiamine-S^{35} of labeled TTP, TPP, and TMP. The starting thiamine and the phosphorus esters synthesized from it contained radioactive sulfur with the same specific activity, which made much easier the later dosage of the resulting vitamins according to their radioactivity, and also simplified the design of the isotopic investigation in animals. The method of Viscontini was modifie by us for use with small amounts of initial thiamine-S^{35}. Fractionation of the thiamine phosphorus esters, in distinction from the original method, was carried out by electrophoresis. Below we describe the method of obtaining a mixture of the phosphorus esters of thiamine-S^{35}.

Phosphorylation of thiamine-S^{35}. Four ml of 83% orthophosphoric acid was heated in a tube of heat resistant glass over an open flame until it became turbid. The viscous mixture of polyphosphoric acids was cooled to 120° on an oil bath and about 0.5 ml of it was added to a centrifuge tube which contained 5-40 mg of thiamine-S^{35}. The polyphosphoric acids quickly thickened when cooled; the mixture of thiamine-S^{35} and polyphosphoric acids in the centrifuge tube was placed in a bath with vaseline oil at 105° and constantly stirred with a glass rod. After 15 min the light brown reaction mixture was removed from the oil bath, cooled in air, and then with a stream of tap water.

Separation of phosphorus esters of thiamine-S^{35} as phosphates. To the thick reaction mixture was added 0.5 ml of a 1% solution of orthophosphoric acid cooled to 0°, the mixture was carefully sitrred with a glass rod and centrifuged for 5 min at 3000 rpm. The clear extract

TABLE 1. Yield and Loss in Different Stages of the Synthesis of Phosphorus Esters of Thiamine-S^{35} in %

Thiamine-S^{35} taken, mg	Radioactivity of fraction				
	I	II	III	IV	B_1 esters
20,1 (22 916)	1,3 (312)	9,0 (2 070)	1,7 (399)	0,2 (40)	87,8 (20 095)
6,6 (51 668)	0,6 (351)	7,8 (4 179)	2,9 (1 539)	0,1 (75)	88,6 (45 524)
22,0 (94 606)	1,8 (1 734)	4,3 (4 132)	0,9 (859)	0,1 (141)	92,9 (87 696)
6,0 (29 789)	0,3 (88)	6,5 (1 953)	0,5 (156)	0,2 (77)	92,5 (27 515)
39,4 (254 096)	1,8 (4 665)	5,0 (12 797)	2,0 (3 150)	0,3 (884)	90,9 (232 600)

was poured into a large centrifuge tube and the residual precipitate was twice more extracted with the same solution of orthophosphoric acid. The remaining protein precipitate, insoluble in cold 1% orthophosphoric acid, was discarded (I). The extracted phosphates of the phosphorus esters of thiamine-S^{35} in a small volume (about 1.5 ml) were precipitated by the addition of 40 ml of dry acetone cooled to −10 to −15°. The cloudy solution with acetone was put in a refrigerator for two hours for more complete precipitation, and was then centrifuged twice for 30 min, placing the centrifuge vessel in the refrigerator in the interval between centrifuging. Thereupon, on the bottom of the centrifuge vessel and often on the walls there formed a small amount of colorless, oily precipitate. The almost clear acetone, which contained the residue of unprecipitated esters of thiamine-S^{35}, was discarded (II).

Separation of phosphorus esters of thiamine-S^{35} as hydrochlorides. To the oily precipitate collected on the bottom and walls of the centrifuge vessel was added 1 ml of 2 N HCl and 3 ml of acetone; this mixture dissolved the precipitate adhering to the walls. The resulting hydrochlorides of the phosphorus esters of thiamine-S^{35} were precipitated with 40 ml of dry acetone as in the preceding case. It should be noted that these salts of the phosphorus esters of thiamine are precipitated more rapidly and clear acetone is obtained even after 30 min centrifuging. The acetone was discarded (III) and the dense, sticky precipitate was washed with a mixture of absolute alcohol and dry acetone (1 : 1) cooled to −10 to −15° and then with dry, cooled ether; the precipitate was stirred up with a glass rod and centrifuged for 20 min at 3000 rpm. The supernatant liquid was discarded (IV). The resulting phosphorus esters of thiamine-S^{35} appeared as a white, hygroscopic powder on the bottom of the centrifuge vessel and were kept in this form in a vacuum desiccator in the dark at a temperature of 2°.

Working with labeled thiamine-S^{35} we were able to determine accurately the percent yield of phosphorus esters and also to calculate the loss of the vitamin at all stages of the synthesis. The results of these studies are given in Table 1. The yield of hydrochlorides of the phosphorus esters of thiamine-S^{35} when the above described method was used was on the average 90.5% (87.8-92.9%) of the radioactivity of the initial thiamine-S^{35}. As for the loss of radioactive sulfur of thiamine-S^{35} in the different stages of the synthesis, it should be noted that in the insoluble precipitate I which remained after extraction of the phosphorus esters from the reaction mixture there was 0.3 to 1.8% of the radioactivity. The main loss is connected with incomplete precipitation of the phosphates of the phosphorus esters of thiamine-S^{35} by acetone, II. In this fraction we discarded from 4.3 to 9.0% of the radioactivity. In the second precipitation of the hydrochlorides of the phosphorus esters of thiamine-S^{35} with acetone III a part of the vitamin is also discarded (0.5-2.9%) because of incomplete precipitation. The loss in washing the precipitate of phosphorus esters of thiamine-S^{35} with a mixture of alcohol and acetone and with ether is very small (0.1-0.3%).

In one control experiment on the synthesis of phosphorus esters of thiamine by the above method we used labeled phosphate (P^{32}), 0.1 ml of a 0.4 solution of which was added to 4 ml of 83% orthophosphoric acid. After heating there was formed a mixture of labeled polyphosphoric acids (0.5 ml) which was used for phosphorylation of 10.0 mg of nonradioactive vitamin B_1. The distribution of radioactive phosphorus in the fractions was the following:I) 82.8; II) 16.7; III) 0.002; and IV) 0.0007%. The resulting mixture of phosphorus esters of thiamine showed 0.43 % of the radioactive phosphorus. The distribution of radioactive phosphorus in the fractions showed that most of the polyphosphoric acids were not extracted from the reaction mixture along with the phosphorus esters of thiamine, but remained in precipitate (I). Those phosphoric acids which were extracted with the vitamin were almost completely removed by acetone (II) in the precipitation of the phosphorus esters of thiamine. The later reprecipitation (III) and washing (IV) almost completely washed the phosphorus esters from free phosphates.

Thus, our modification of the Viscontini method [13], assures a high percent yield (90.5%) of the phosphorus esters of thiamine-S^{35} and can be used successfully for work with small amounts of vitamin B_1.

Identification and Quantitative Determination of the Resulting Phosphorus Esters of Thiamine-S^{35}

In order to identify and determine quantitatively the synthesized phosphorus esters of thiamine-S^{35} it was essential to separate them from each other. The suggestion of Viscontini and coworkers [13] of fractionation of phosphorus esters of thiamine by their precipitation

TABLE 2. Effect of Ionic Strength of Buffer Solutions, Amount and Place of Deposition of Thiamine-S^{35} Esters on Their Electrophoretic Mobility

Studied component	Place of deposition in mm (from anode)	Vitamin, µg	Concentration of formic acid µ***	pH at 10°	Current strength ma/cm	Migration in mm from point of deposition to the cathode	to the anode		
Thiamine*	225	20	0,1	2,69	0,20	130			
	100	20	0,1	2,69	0,20	195			
	100	40	0,1	2,69	0,20	195			
	100	80	0,1	2,69	0,20	195			
	100	160	0,1	2,69	0,20	150			
	100	320	0,1	2,69	0,20	105			
	100	80	0,05	2,84	0,16	220			
	100	80	0,025	2,99	0,12	225			
TMP**	225	20	0,1	2,69	0,20	65			
	100	20	0,1	2,69	0,20	90			
	100	40	0,1	2,69	0,20	90			
	100	80	0,1	2,69	0,20	90			
	100	160	0,1	2,69	0,20	65			
	100	320	0,1	2,69	0,20	45			
	100	80	0,05	2,84	0,16	110			
	100	80	0,025	2,99	0,12	120			
TPP**	225	20	0,1	2,69	0,20	13			
	100	20	0,1	2,69	0,20	25			
	100	40	0,1	2,69	0,20	25			
	100	80	0,1	2,69	0,20	23			
	100	160	0,1	2,69	0,20	15			
	100	320	0,1	2,69	0,20	12			
	100	80	0,05	2,84	0,16	28			
	100	80	0,025	2,99	0,12	35			
TTP**	225	20	0,1	2,69	0,20		7		
	100	20	0,1	2,69	0,20	14			
	100	40	0,1	2,69	0,20	12			
	100	80	0,1	2,69	0,20	12			
	100	160	0,1	2,69	0,20	10			
	100	320	0,1	2,69	0,20	7			
	100	80	0,05	2,84	0,16	7			
	100	80	0,025	2,99	0,12	5			
Thiamine tetraphosphate (I)	225	20	0,1	2,69	0,20		*I*-25; *II*-35; *III*-65		
Thiamine penta (Hexa) phosate (II)	100	20	0,1	2,69	0,20		12	23	35
	100	40	0,1	2,69	0,20		12	21	35
Thiamine poly (?) phosphate (III)	100	80	0,1	2,69	0,20		8	17	34
	100	160	0,1	2,69	0,20		8	13	15
	100	320	0,1	2,69	0,20		6	6	7
	100	80	0,05	2,84	0,16		8	17	25
	100	80	0,025	2,99	0,12		8	15	23

*Identified by deposition on a control strip of thiamine.

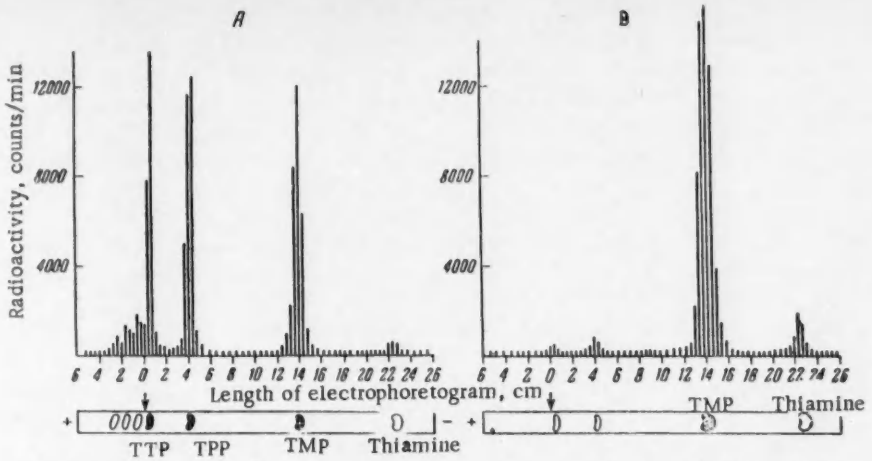
**Identified by the radioactive method.

***For formic acid µ equals the molarity [26].

from water solution with increasing amounts of acetone is useful only with respect to relatively large amounts of phosphorus esters of thiamine to be precipitated. Besides, our investigation showed that such fractionation does not give pure preparations of TTP, TPP, and TMP. The only hopeful processes for the separation of the phosphorus esters of thiamine from their mixture are their chromatographic or electrophoretic separations.

The first work on separation of phosphorus esters of thiamine by paper chromatography was published in 1953. A mixture of phosphorus esters of thiamine was placed on chromatographic paper in small amount (10-100 µg) [17]. For the separation, different acid separating mixtures were used: n-butanol-formic acid (82 : 18) [18]; n-propanol-water-1 M acetate buffer, pH 5.4 (65 : 20 : 15) [15] or (70 : 20 : 10) [19], and others.

For the electrophoretic separation, various acid buffer solutions were used: 0.1 M NaCl-glacial acetic acid (1000 : 3,5) [20]; acetate buffer pH 5,4 [15]. The chromatograms and electrophoretograms were developed by spraying them with an alkaline solution of ferricyanide [18]. For quantitative determination of the separated phosphorus esters of thiamine different methods were used: manometric determination of cocarboxylase activity [19]; colorimetric or densitometric determination of phosphorus in the different thiamine esters; measurement of the radioactivity of thiamine-S^{35} or phosphorylated P^{32} [17]; photometric measurement of the darkening of a film after



Electrophoretogram of a mixture of phosphorus esters of thiamine-S^{35} (A) and the hydrochloric acid hydrolyzate of this mixture (B). On the electrophoretogram is shown the fluorescence in ultraviolet light of the phosphorus esters of thiamine.

TABLE 3. Identification of P^{32} Phosphorus Esters of Thiamine-S^{35} on the Electrophoretogram (for the separation we used 80 μg of the mixture of P^{32} phosphorus esters of thiamine-S^{35})

Distribution of fractions from the cathode	Character of fluorescence	Counts/min		Multiple ratio, counts/min		Identified compound
		P^{32}	S^{35}	S^{35}	P^{32}	
1	Weak	0	1750	–	–	Thiamine*
2	"	0	320	–	–	Thiochrome**
3	Clear	250	9440	37.76	1.00	TMP***
4	"	804	15,691	37.76	1.93	TPP
5	"	1096	13,135	37.76	3.15	TTP
6	Weak	820	8040	37.76	3.85	Thiamine tetraphosphate
7	"	278	1939	37.76	5.41	Thiamine penta (hexa) phosphate
8	"	27	115	–	–	Thiamine polyphosphate****

*Identified by deposition on a control strip of thiamine-S^{35} in the absence of P^{32}, and by development of a blue fluorescence after treating the electrophoretogram with an alkaline solution of ferricyanide.

**Identified by deposition on a control strip of thiochrome-S^{35}, and also in the absence of P^{32}, and by the blue-violet fluorescence on the untreated electrophoretogram.

***Identified by stability to acid hydrolysis.

****Unidentified polyphosphate esters of thiamine.

TABLE 4. Quantitative Determination and Standardization of Eluates of Thiamine, TMP, TPP, and TTP Obtained from Thiamine-S^{35} of the Same Specific Activity (1 μg – 1484 counts/min)

Eluted component	Volume of eluate, ml	Counts/min, 0.1 ml of eluate	Volume of eluate containing 100 μg vitamin, ml	To obtain 5 mg% solution, eluate diluted
Thiamine	25.0	796	1.86	1.07 times
TMP	22.5	1140	1.03	1.94 "
TPP	11.0	2662	0.55	3.60 "
TTP	14.0	1779	0.83	2.40 "

photography of the fluorescence of the chromatogram [21, 22]; measurement of the fluorescence of thiochrome after splitting the phosphorus esters of thiamine with takadiastase [23]; direct photometric determination of the phosphorus esters of thiamine in the Beckman spectrophotometer [24].

We were convinced by the use of various methods that the phosphorus esters of thiamine could be separated by paper chromatography and the best results were obtained by using the mixture of Siliprandi [15]. However, we believe that with paper electrophoresis the phosphorus esters of thiamine are separated much faster and better and the distance between the different components is considerably greater than in chromatography. In studying the ammonia-formate buffer suggested by Rossi-Fanelli and coworker [16] we noted that the use of formic acid alone instead of this buffer gave very much better separation of the phosphours esters of thiamine. This fact considerably simplifies the method of separation of the phosphorus esters of thiamine-S^{35}.

The synthesized mixture of phosphorus esters of thiamine-S^{35} was dissolved in distilled water to give a 400-800 mg% solution with pH 4.5-5.0. Such solutions were kept in the dark at 2°. For the electrophoretic separation of the studied mixtures the phosphorus esters of thiamine-S^{35} were deposited on seven strips of chromatographic paper (each 2 cm wide) and they were moistened with freshly prepared formic acid solution (0.1-0.025 M) and immersed in the same buffer solution (2 liters) in a moist electrophoretic chamber. The length of the nonimmersed part was 45 cm, and after electrophoresis, 30 cm. In the final variant, the removable horizontal part of the phoretogram, length 32 cm, after it had been moistened, was laid on a strip of dense chromatographic paper dipping into the buffer solution. The voltage was 11 v/cm, temperature 10-12° (with external cooling). Time of separation, 4 hours. The electrophoretic mobility of the phosphorus esters of thiamine-S^{35} under these conditions depended on the point of deposition of the mixture, the quantity of components deposited, and also on the ionic strength of the formic acid (Table 2).

The points of separation and the amount of the separate phosphorus esters of thiamine-S^{35} were determined by the method of radioactive indicators according to our method [25] and also by the distribution of fluorescing spots in filtered ultraviolet light (340 mμ), after developing the phoretogram with an alkaline solution of ferricyanide.

The experiments showed that electrophoretic separation of the phosphorus esters of thiamine is considerably improved by depositing the mixture on the strip close to the anode (10 cm from the surface of electrolyte). In this case there is an increase in the electrophoretic mobility of thiamine, TMP, and TPP. When the mixture of esters is deposited in the middle of the electrophoretic strip, the mobility of thiamine, TMP, and TPP is less and the separation becomes poorer. However, this improves the separation of the polyphosphate esters of thiamine and increases their electrophoretic mobility. Also, when deposition is in the middle the effect of movement of the buffer solution along the strip on the electrophoretic mobility of the studied components is excluded [27], and conditions are created for determining the isoelectric points of some of them. With such an arrangement of the experiments we showed that TPP, TMP, and thiamine are cations and TTP, thiamine tetraphosphate, and the other polyphosphate esters of thiamine are anions. Judging by the electrophoretic mobility, at the pH used TTP and to a lesser degree TPP are close to their isoelectric points, which agrees with the literature data [24, 28]. The amount of phosphours esters of thiamine deposited on the separate strips also has a very great effect on the sharpness of their separation and electrophoretic mobility. The more of the mixture of phosphorus esters of thiamine deposited, the worse is their separation. When on one strip there is deposited 320 μg of the mixture, only three fractions are found (thiamine, TMP, and all the rest in one fraction). With deposition of 160 μg of mixture, five fractions are found (thiamine, TMP, TPP, TTP, and polyphosphates), which are so close to each other that their determination is difficult. When 80 μg are deposited, there is complete separation of the phosphorus esters of thiamine-S^{35} which make up the mixture. Interesting results are obtained by decreasing the ionic strength of the formic acid from 0.1 M to 0.025 M, which considerably improves the separation and electrophoretic mobility of thiamine, TMP, and TPP. After many experiments we found the optimum conditions for separating a mixture of phosphorus esters of thiamine in a moist chamber simultaneously on seven strips; 80-100 μg of ester mixture are deposited on each strip 10 cm from the anode and separation is carried out for four hours in 0.025 M formic acid at a potential gradient of 11 v/cm, temperature 10-12°. These conditions of electrophoresis (used in all the following experiments) give well reproducible results.

The identification of the separate phosphorus esters of thiamine on the electrophoretogram was carried out by two methods. At first we studied the stability of the separate phosphorus esters of thiamine toward acid hydrolysis. For this purpose we deposited on parallel strips 80 μg of the studied mixture of phosphorus esters of thiamine-S^{35} before and after its 10 min hydrolysis by 2 N HCl at 100°. The results of one of these experiments are given in the figure.

In studying the quantitative composition of the separate phosphorus esters of thiamine-S^{35} on the electrophoretogram after 10 min hydrolysis in 2 N HCl at 100°, we observed transformation of all the phosphorus esters into one phosphorus ester of thiamine. According to the literature data [10, 13, 28] only TMP is not hydrolyzed under these conditions. On the basis of these data we have identified TMP.

We carried out more detailed identification of all the phosphorus esters of thiamine using the isotope method. We synthesized the P^{32} phosphorus esters of thiamine-S^{35} by the method described above. The resulting mixture of P^{32} phosphours esters of thiamine-S^{35} was separated electrophoretically. The electrophoretogram was counted under an end counter along the whole length and was developed by an alcoholic alkaline solution of ferricyanide. The observed radioactive and fluorescing components were outlined in pencil and cut out with the corresponding parts of the electrophoretograms. In each of the thus isolated components we counted the content of P^{32} and S^{35} with an end counter. For calculation of the radioactive phosphorus without the radioactive sulfur we used an aluminum filter which covered the mica window of the end counter. The circular aluminum filter (diameter 3 cm) with thickness 0.25 mm was cut from the tube of a β-counter of type B-2. It was shown in the preliminary experiments that such a filter absorbs 15.3% of the β-radiation of P^{32} and 100% of the β-radiation of S^{35} in counting with an end counter. The phosphorus esters of thiamine on the electrophoretogram were identified in the studied components by the ratio of P^{32} and S^{35}. The possibility in principle of such identification of the phosphorus esters of thiamine was shown by Shnol' [17].

However, we considered it necessary to describe in detail the method and calculation in an example from one experiment, the results of which are given in Table 3. After counting the radioactivity of P^{32} and S^{35} in each of the components studied and considering that in the TMP identified by us in a previous experiment for each atom of S there is one atom of P, we calculated the number of counts/min/atom S^{35}; if 1 atom of P^{32} gives 1 count/min; this came out 37.76 counts/min. Starting from this we calculated how many atoms of P^{32} occurred for each atom of S^{35} in the remaining P^{32} phosphorus esters of thiamine-S^{35}.

Calculation of the multiple ratio gave results which agreed well with the theoretically possible ones (1.93 atoms P^{32} in TPP, 3.15 atoms P^{32} in TTP, and 3.85 atoms P^{32} in thiamine tetraphosphate). For the residual polyphosphate esters of thiamine it was impossible to carry out exact calculations because of the small content of them in the synthesized mixture of phosphorus esters of thiamine.

In the mixture of phosphorus esters of thiamine-S^{35} synthesized by the method described above, counted on the radioelectrophoretogram, the following were noted in percent; thiamine 2.5 (0.8-3.3); TMP 27.8 (22.0-31.4); TPP 30.6 (26.6-39.9); TTP 30.0 (26.7-33.8); thiamine tetraphosphate 6.9 (4.2-10.5). There was less than 3% of the radioactivity in the remaining polyphosphate esters of thiamine-S^{35}. When 200-400 mg% solutions of the mixture of phosphorus esters of thiamine-S^{35} (pH 4.5-5.0) were kept in the dark at 2° for a month, no change was observed in the quantitative composition of the main fractions if we do not consider the appearance of a small amount of thiochrome due to oxidation of thiamine.

Obtaining Eluates of the Phosphorus Esters of Thiamine-S^{35} from the Electrophoretograms and Their Standardization

In order to obtain the phosphorus esters of thiamine-S^{35} in pure form and in amounts sufficient for animal experiments we used their eluates from the radioelectrophoretogram. In two moist chambers we put 14 strips of chromatographic paper simultaneously (on each strip we deposited 80-100 μg of the mixture of the phosphorus esters of thiamine-S^{35}). After the electrophoresis and drying, each electrophoretogram was counted with an end counter provided with a cross slit aluminum window (2 × 20 mm) to observe the boundaries of distribution on the spectrophoretogram of TMP, TPP, and TTP. Then the portions of the electrophoretogram corresponding to TMP, TPP, and TTP were cut out and kept in a vacuum desiccator in the dark at 2°. The phosphorus esters of thiamine-S^{35} prepared over two-three days were eluated from the corresponding part of the electrophoretogram with freshly prepared twice distilled water. For this purpose the portions of the electrophoretogram which corresponded to one of the phosphorus esters of thiamine. were fastened with thread into a strip with a length of about 50 cm. In a special dark chamber we added at the upper end of the strip from a microburet drops of twice distilled water and we collected the eluate in a small flask. Elution was continued for two hours, after which a considerable part of the water was evaporated, and the small volume of eluate washed 60 to 80% of the vitamin from the strip. The content of phosphorus esters of thiamine-S^{35} varied in the eluates from 3.9 to 7.5 mg% calculated on thiamine-S^{35}; the pH of the resulting solutions varied from 5.0 to 6.0. Using the electrophoretic method described above we also purified thiamine-S^{35} from its accompanying contaminants and obtained its pure solution as an eluate (the time of electrophoresis here was decreased to three hours).

Quantitative determination of the content of thiamine, TMP, TPP, and TTP in the resulting eluates was carried out by measuring their radioactivity which was compared with the radioactivity of standard solutions containing 1 μg of starting thiamine-S^{35} of the same specific activity. The results of the experiments are given in Table 4.

In this experiment we obtained 5.0 mgμ solutions of thiamine-S^{35} and sulfur-labeled TMP, TPP, and TTP (calculated on the thiamine). Such standarized solutions, containing pure components, were injected parenterally into various animals for comparative study of their metabolism. During the period of use (2-6 days) the standardized solutions of thiamine and its phosphorus esters were kept in the dark at 2°.

SUMMARY

We have presented a micromethod for obtaining pure solutions of phosphorus esters of thiamine, labeled on sulfur, from a small amount of initial thiamine-S^{35} (5-40 mg).

The phosphorus esters of thiamine-S^{35} were obtained in the form of a mixture of TMP, TPP, TTP, thiamine tetraphosphate, and other polyphosphate esters of thiamine-S^{35} by a modification of the method of Viscontini, Bonetti, and Karrer [13]. The resulting mixture of phosphorus esters of thiamine was separated by electrophoresis. Identification of the thiamine esters on the electrophoretogram was carried out with specially synthesized compounds with double labeling by P^{32} and S^{35}.

The phosphorus esters of thiamine-S^{35} detected by radioactivity were obtained in the form of pure aqueous solutions by their elution from the corresponding portions of the electrophoretogram.

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THE DECREASE IN STABILITY OF MYOSIN IN SOLUTION AND ITS ADENOSINETRIPHOSPHATASE ACTIVITY IN THERMAL DENATURATION AND UNDER THE INFLUENCE OF CADMIUM CHLORIDE

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The decrease in adenosinetriphosphatase activity of myosin can occur because of reversible or irreversible changes in the state of the protein. Decrease in ionic strength of the solution, shift in pH from 8.0 to 7.0 [1], irradiation in small doses [2], action of cadmium chloride in low concentrations [3] lead to reversible decrease in enzymatic activity of myosin. In the latter case there is also a reversible decrease in the number of free SH groups of the protein and its stream birefringence. Irreversible inactivation of myosin occurs with acidification to pH below 6.0 and at pH 10, under the influence of large concentrations of $CdCl_2$ [4] and with thermal denaturation [1]. The latter should be related to other irreversible changes since it is accompanied by splitting of the protein into fragments which have deaminase activity [5] and are water soluble [6]. On the basis of these results we can assume that different agents produce destruction of the myosin structure of different depth and character, such as are also found for a number of other proteins (for example, for urease [7], ribonuclease [8], chymotrypsin [9], and serum albumin [10]).

We have tried to distinguish such changes experimentally in the denaturation of myosin by heat and by cadmium chloride, detecting them by two quantitative indexes: ATP-ase activity and decrease in stability of the protein in solution. It is known that myosin solutions at pH < 6.6 and containing more than 3-4 mg/ml of protein are coagulated on heating. However, at pH ≥ 6.6 fully or partially thermal inactivated myosin in 0.5 M KCl does not give a precipitate. With progressive lowering of the salt concentration, the protein with lower solubility precipitates from such solutions before the native protein and separately from it. The possibility of such separate precipitation of denatured myosin by dilution was used by us for its quantitative determination.

METHODS

Myosin was extracted for 10 min from ground rabbit muscle with a cooled Bailey solution and was precipitated by twentyfold dilution of the clear filtered extract with twice distilled water at pH 6.8. After a second reprecipitation (also by twentyfold dilution), a solution of the myosin in 0.5 M KCl, which contained 15-20 mg/ml of protein and had Q_p = 3000-4000, was kept at 2-4°. The ATP-ase activity of this solution as a rule did not decrease during 14-15 days. Its DLP did not fall when 5 mg ATP was added per 1 mg myosin; the preparation was homogeneous on sedimentation in the ultracentrifuge [11]. Some of the main experiments were also carried out with myosin freed from actin by the method of Szent-Györgyi, but without "crystallization". The protein was extracted with Bailey solution and precipitated by tenfold dilution, then dissolved in 0.02 M K_2CO_3 and brought to pH 8.3 with phenolphthalein. Then we added 4 ml of 2 M KCl for each gram of protein and 50 ml of water at pH 8.3 for each milliliter of 2 M KCl added. The actomyosin gel insoluble at 20° was separated by centrifuging; myosin was precipitated by twofold dilution of the centrifugate with water at pH 6.8 and was dissolved in 0.5 M KCl. The myosin obtained after three reprecipitations by fifteen fold dilution gave a solution in 0.5 M KCl without opalescence and one which had Q_p = 1800-2700.

The protein concentration in the starting solution was determined by weighing, precipitating it by thermal coagulation in 70% alcohol and then washing with water and drying to constant weight at 115°.

For determination of the ATP-ase activity of the myosin a sample containing 0.3 ml of 0.05 M borate buffer (pH 9.2), 0.1 ml of 0.05 M $CaCl_2$, 0.09 mg myosin (in a volume of 0.3 ml), and 1.5 mg ATP (in a volume of 0.3 ml) was kept for 10 min at 37° and then 1 ml of 10% trichloroacetic acid was added. Phosphorus was determined in the filtrate by the Fiske-Subbarow method. Special experiments showed that the amount of phosphorus split by the myosin was exactly proportional to the time of incubation within the limits from 5 to 13 min.

All the experiments were carried out with myosin solutions diluted to 3 mg/ml (except the experiment given in Fig. 1) in 0.5 M KCl (solution I), in Bailey solution (II), in 0.05 M borate buffer, pH 7.8, in 0.5 KCl (III), in 0.5 M KCl with 0.1 M acetate added to a final pH of 6.1 of the protein solution (IV), and with a solution of myosin freed from actin in a borate buffer, pH 7.8, in 0.5 M KCl (IIIa). The pH values were determined with an LP-5 potentiometer with a glass electrode. The protein solutions were heated in a water thermostat kept at the required temperature with an accuracy of ± 0.03°.

The protein concentration in the centrifugate sample was determined by one of two methods: by light absorption in the ultraviolet measured on an SF-4 spectrophotometer and by the color reaction of Lowry [12]. The first method gives satisfactory results if we introduce a correction for light scattering in the ultraviolet for 276 mμ, ϵ_T, calculating its value from the optical density for 276 mμ. Calculation of ϵ_T by the Rayleigh formula from the value of ultraviolet absorption for 320 and 360 mμ, ϵ_{320}, and ϵ_{360}, for eighteen myosin preparations showed that there was a sufficiently constant ratio $\epsilon_T / \epsilon_{320} = 1.53$. Therefore, instead of calculating ϵ_T we used a correction equal to $\epsilon_{320} \cdot 1.53$ and obtained the true absorption with a relative error not above 2%. For a series of dilutions of myosin at 0.5 M and 0.25 M KCl we obtained a linear relation of the value of $\epsilon_{276} - \epsilon_{320} \cdot 1.53$ to the protein concentration in the interval 0.1-1 mg/ml protein. The calibration curve by the method of Lowry also gave a linear relation of ϵ with the concentration of myosin in the limits from 0.01-0.3 mg/ml of protein.

Precipitation of the protein: The desired rate and completeness of precipitation of myosin, altered by heating, was obtained only at pH 6.8-7.0; therefore the conditions of precipitation depended on the pH of the initial solution of the protein and this was determined for each solution separately. Solution I (pH 8.0) was kept at 37° for four hours after which the ATP-ase was fully inactivated. Then to a series of samples of heated and native protein we added progressively increasing amounts of 0.01 M, phosphate buffer, pH 6.4. After keeping in the cold for not less than four hours the precipitates in all the samples were separated by centrifuging; we determined the protein content in the centrifugate and expressed the concentration of unprecipitated protein in the heated samples in percent of the protein in the native samples of corresponding dilution. The relation of these values to the concentration of KCl is shown for a solution of 1 mg/ml of protein (Fig. 1). We see that in the KCl concentration interval 0.25 M-0.15 M 71% of the heated protein precipitates, while in the native samples practically all the protein remains in solution. Heated solutions II (ph 8.6), III and IIIa (pH 7.8) were precipitated with 0.01 M phosphate buffer, pH 5.8-6.0 or with 0.01 M acetate, pH 4.7; solution IV (pH 6.1) was precipitated with 0.01 M phosphate buffer, pH 7.4. Full precipitation resulted with 0.21-0.24 M KCl at pH 6.8. As a rule a slight amount of precipitate also settled out of the unheated solutions in the form of light flakes (probably actomyosin, since solution IIIa did not give such a precipitate). In the heated solutions we determined the percent content of protein nitrogen with unchanged stability in the solution. For this we used the calibration curve of precipitation for each solution separately. For this we set up three mixtures of native and thermally inactivated solutions in the ratios 1 : 3, 1 : 1, and 3 : 1. To five samples which contained 100, 75, 50, 25% native and 100% thermally inactivated protein we added 0.01 M buffer in the amount needed for complete precipitation and found the concentration of protein in the centrifugate. From the first four values of the concentration we calculated it in the fifth sample. The first number obtained was the concentration of protein precipitated in full thermal inactivation and was taken as 100%. The relation of the content of protein which remained in solution (in % of the precipitated), N_1 from the percent content of native protein N gave the calibration curve, from which, knowing N_1 in a given solution we found the value of $N = c/c_0 \cdot 100$, where c and c_0 are concentrations of native protein in the heated and original solutions.

RESULTS

Solutions I and II which stood from 1 to 12 days in the cold after purification, were kept for different intervals of time from 10 to 30 min at 37°, after which we

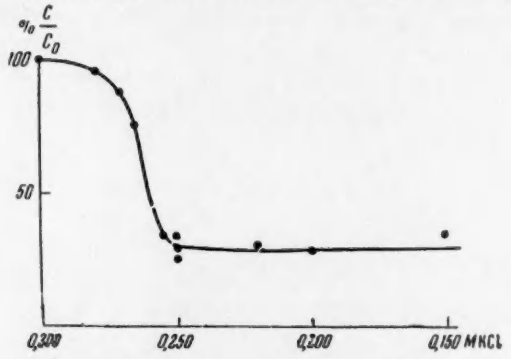

Fig. 1. Relation of solubility of thermally inactivated myosin to KCl concentration. Initial myosin solution 1 mg/ml heated 4 hours at 37°. On the abscissa, molar concentration of KCl. On the ordinate, unprecipitated protein in % C/C_0 where C and C_0 are the concentrations of unprecipitated protein corresponding to heated and unheated solutions, determined by ultraviolet absorption.

determined the ATP-ase activity and content of native protein in the samples. The results of measurement for 13 solutions are given in Fig. 2. The scatter of points was caused to a considerable extent by different "ages" of the solutions. The thermal stability of ATP-ase falls with period of keeping (rate of thermal inactivation rises); on the contrary, the precipitability of the protein is worsened. The resulting curves of course of thermal inactivation and precipitation do not give a linear relation on a semilogarithmic scale. The same measurements carried out on the same solution during one day give a relation for both indexes with time close to exponential; this is seen for solution III at three temperatures of heating on the sixth, seventh, and eighth day of keeping (Fig. 3). The experiments with solution IIIa ("without actin") gave the same results (Table 2).

The rapid increase in the amount of precipitating protein in the first 15-20 min of heating can be explained

TABLE 1. ATP-ase Activity of Precipitated and Nonprecipitated Fractions of Heated Myosin Solutions

Fraction	Preparation, time of heating, temperature	Q_p	Activity, %	Amount of protein, % of protein of sol.	Splitting of protein, % of protein of solution. Calc. from activity		Preparation, time of heating, temperature	Q_p	Activity, %	Amount of protein, %, of protein of sol.	Splitting of protein, % of protein of solution. Calc. from activity	
					centrifugate	precipitate					centrifugate	precipitate
Heated solution			95.7						94.2			
Centrifugate	III, 15'	3650	84.5	80.3	12.4	8.9	III, 15',	3730	47.0	51.0	27.0	–
Precipitate	37°		106.5	19.7			37°		77.0	49.0		
Heated solution			99.0						68.0			
Centrifugate	III, 15',	3700	68.5	68.0	21.5	–	III, 20',	4400	50.0	35.0	17.5	26.6
Precipitate	37°		99.0	32.0			37°		71.5	65.0		
Heated solution			63.7						92.3			
Centrifugate	III,20',	4200	77.8	37.9	8.4	20.0	III, 20',	3650	35.9	36.5	23.4	–
Precipitate	37°		55.3	62.1			37°		79.5	63.5		
Heated solution			91.4						67.0			
Centrifugate	III, 20',	3730	33.4	31.6	21.1	–	III, 30',	3950	36.2	19.3	12.6	–
Precipitate	37°		70.0	68.4			37°		22.6	80.7		
Heated solution			62.2						62.8			
Centrifugate	III, 30',	3650	33.0	24.4	16.3	9.0	III, 30',	3600	37.5	23.0	14.4	–
Precipitate	37°		55.8	75.6			37°		80.0	77.0		
Heated solution			85.3						39.8			
Centrifugate	III, 45',	3750	44.0	33.5	18.7	6.5	III, 60',	3540	6.0	11.8	11.1	–
Precipitate	35°		83.0	66.5			37°		55.0	88.2		
Heated solution			78.1						67.0			
Centrifugate	IIIa,	1860	76.8	43.6	–	–	IIIa,	1970	10.2	51.1	–	–
Precipitate	15', 37°		68.2	56.4			20',37°		61.3	48.9		

Note: Myosin concentration 3 mg/ml in 0.5 M KCl with borate buffer pH 7.8. Solution III – twice precipitated myosin; determination of concentration by ultraviolet absorption; precipitate not washed. Solution IIIa – three times reprecipitated myosin, freed from actin; determination of concentration according to Lowry; precipitate twice washed with 0.22 M KCl.

by the presence in the unheated solution of a considerable amount of inactive protein which retains its stability in solution, but rapidly loses this at the beginning of heating without notably lowering the ATP-ase activity of the whole sample. This suggestion is not justified. As a test for it we determined in a number of experiments after precipitation of the protein the ATP-ase activity of the centrifugate and the separated precipitate, not washed, or washed twice with 0.22 M KCl and dissolved in 0.5 M KCl. In these experiments precipitation was carried out with an acetate buffer; the KCl concentration in the centrifugate was brought to 0.5 M. Table 1 shows that the activity of the protein in the precipitate, as a rule, is higher than in the centrifugate, and is close to the activity of the heated solution.

The lower activity of the centrifugate as compared to the precipitate can indicate a splitting from the precipitating protein of a fraction which lacks ATP-ase activity and which enters the solution. The splitting of such a protein fragment from myosin in thermal denaturation is well known [5, 6]. In our experiments the portion of nonprecipitating protein in complete thermal inactivation varies from 8-9% to 30-31%; its amount evidently increases with pH and with time of keeping the protein solution. Values of the same order are given by attempts to calculate the portion of splitting protein with incom-

TABLE 2. Thermal Inactivation and Decrease in Stability in Myosin Solutions at 37°, Free from Actin (myosin concentration 3 mg/ml; solution IIIa; concentration determined according to Lowry)

Q_p	Time of heating, min	Activity, % Q_p	Native protein, %
2750	10	98,2	63,5
	15	87,6	50,5
	30	69,4	36,5
2300	12	78,9	44,5
	20	76,0	24,5
	40	44,9	4,0
	60	32,0	<0,5

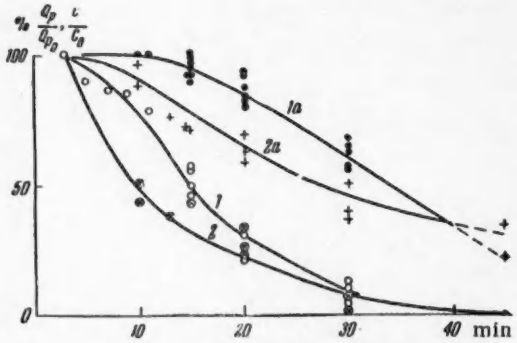

Fig. 2. Fall in ATP-ase activity in solutions of myosin in 0.22-0.24 M KCl at 37°. 1 and 1a – 3 mg/ml myosin in 0.5 M KCl, pH 8.0; 2 and 2a – the same in Bailey solution, pH 8.6. On the abscissa – time of heating; on the ordinate – 1 and 2 amount of native protein in the heated solution (c) in % of the native protein in the original solution (c_0); 1a and 2a, activity in $\%Q_p$.

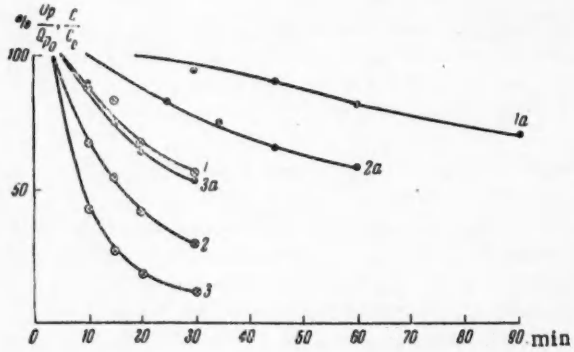

Fig. 3. Fall in ATP-ase activity and solubility of myosin in 0.22 M KCl for one preparation. Myosin 3 mg/ml with borate, pH 7.8; 1 and 1a – native protein and corresponding activity at 35°; 2 and 2a – the same at 37°; 3 and 3a – the same at 39°. Other designations the same as in Fig. 2.

plete thermal inactivation from the activity and protein content in the centrifugate, and in some experiments, also in the precipitate (Table 1). If there is actually an unequal distribution of ATP-ase activity in these fractions from splitting off of inactive fragments, then this splitting, already observed at the beginning of heating, should take place simultaneously with loss of solubility of the protein. This is confirmed to a definite degree by the following experiments: a series of heated samples of solution III were diluted 12 times with water, thus precipitating the native myosin along with the denatured protein, and the ultraviolet absorption of the centrifugate was measured at 276 mμ. The resulting slight concentration of the unprecipitated protein strongly depends on the nature of the coagulation of the precipitate and the results can be considered only roughly orienting. Nevertheless, they show (Fig. 4) that increase in the amount of unprecipitated protein with time does not agree with the course of thermal inactivation.

The results of the experiments with solution IV are given in Fig. 5. Here a lag in thermal inactivation is absent; the latter occurs parallel to the decrease in solubility of the protein. From the experimental points of Figs. 3 and 5 we obtained graphically the relation of logarithm of relative value of activity and concentration of native protein with time of heating for solutions III and IV at three temperatures. From the rate constant values, using the Arrhenius formula, we have calculated graphically the experimental energy of activation, ΔE, for the thermal inactivation and for the reaction of decrease in stability of myosin in solution (Table 3).

We also carried out experiments on the action of cadmium chloride on myosin solutions.

To 3 ml of solution II we added 0.3 ml of a $CdCl_2$ solution to a final concentration of $0.5 \cdot 10^{-4}$ to $2 \cdot 10^{-4}$ M $CdCl_2$; the samples were kept for 60 min at 4°. Then we determined the ATP-ase activity and the percent content of native protein, separating the fraction which was unstable in solution by adding 0.01 M phosphate buffer, pH 6.0; for this we used the data and calibration curves for solution II obtained in the experiments with the action of heat. In parallel with these determinations the solutions of proteins with $CdCl_2$ were precipitated by twelve fold dilution with water and the ATP-ase activity of the precipitated protein was found in 0.5 M KCl solution (Table 4).

Part of the myosin was reversibly inactivated by cadmium chloride, part irreversibly. The portion of the protein which lost its solubility in 0.22 M KCl and the protein irreversibly inactivated were nearly the same, or the first was somewhat lower than the second. The protein inactivated reversibly evidently retained its solubility completely.

DISCUSSION OF RESULTS

The rapid decrease in solubility of the protein at the beginning of heating solutions I, II, III, and IIIa, as in-

dicated, is not connected with the presence in it of protein with decreased thermal stability and low ATP-ase activity. The precipitating fraction has relatively high activity, and another explanation must be sought for the observed differences in rate of thermal inactivation and

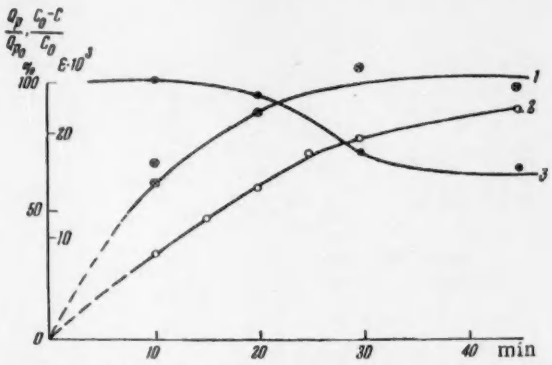

Fig. 4. Splitting at 37° of the myosin fraction soluble in 0.04 M KCl myosin 3 mg/ml in 0.5 M KCl with borate pH 7.8. On the abscissa – time of heating; on the ordinate – 1, extinction of a solution in 0.04 M KCl at 276 mμ ($\epsilon \cdot 10^3$); 2, relative content of denatured protein ($c_0 - c$) in % of native protein in original solution (c_0); 3, ATP-ase activity in $\%Q_p$.

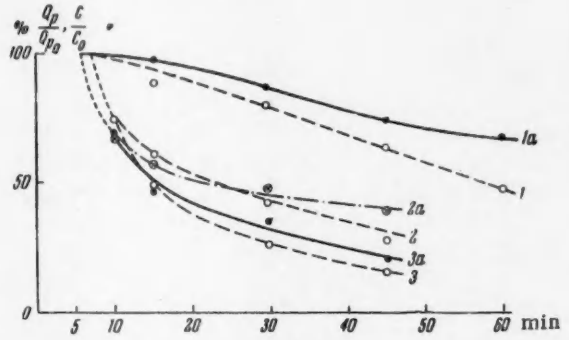

Fig. 5. Decrease in ATP-ase activity and solubility in 0.22 M KCl of myosin heated at pH 6.1. Myosin 3 mg/ml in 0.5 M KCl; 1 and 1a – native protein and corresponding activity at 29°; 2 and 2a – the same at 32°; 3 and 3a – the same at 34°. Other designations the same as in Fig. 2.

decreased solubility. The nonparallel courses of these two processes, and also the data of Table 1, show that in the pH range of greatest stability (pH 7.8-8.6) myosin when heated undergoes two different transformations; for the main mass of protein these occur successively. The first process produces a decrease in solubility of myosin with slight loss of ATP-ase activity or with its full retention. It occurs as a reaction of the first order with an energy of activation of 53 ± 5 kcal/mole. Probably connected with this change in state of the protein is the denaturative splitting of a component which lacks ATP-ase activity and solubility in 0.2-0.25 M KCl.

The sign of the second process is the loss of ATP-ase activity. This reaction is also nearly monomolecular; it occurs at a lower rate, with $\Delta E = 85 \pm 7$ kcal/mole (for solution III) and begins with a marked lag as regards the beginning of heating and compared to the first process. Hence the data for the rate of inactivation (except for the initial points 10 and 15 min for solutions I and II) are obtained for solutions in which not less than 75-80% of the protein has already undergone the first change. Therefore the amount of precipitating protein corresponds to a decrease in ATP-ase activity by 20-25%; therefore, we can also consider that both transformations occur successively for the main mass of the protein. We must also expect that if in denaturation there are two possible and independent transformations, such as two types of rupture of a critical number of hydrogen bonds, they will occur separately but at the same time in two different parts of the protein structure and with lower activation barriers than for the total process. Such a mechanism for the "unfolding of protein molecules in part" was suggested by Mirsky and Pauling [13] for the case of radiation rupture of covalent bonds between the structural particles of native proteins which have the power of configurational changes independent of each other. In our case, with heating, it is possible that the transformations with $\Delta E \cong 53$ kcal/mole and also apparently the total denaturation-inactivation process are probably comparatively slow, since according to our results, a correspondingly energy ΔE should be of the order of 130-140 kcal/mole. Therefore, the greater part of the protein undergoes the first transformation, whose "product", myosin with decreased stability in solution, as it accumulates undergoes thermal inactivation with $\Delta E \cong 85$ kcal/mole. Thus, the initial slowing of thermal inactivation of myosin in the range pH 7-9 shown by Lyubimova [14] is not explained by any inhibition of the reaction; this is actually an induction period which reflects the accumulation of product of the first stage of thermal denaturation.

Has inactivation of ATP-ase already occurred in this first stage? Our results are not sufficient to answer this question. Such inactivation is possible only if the myosin molecule contains more than one center of ATP-ase activity; the data of Mommaerts [15] contradict such a suggestion.

The conclusion that there are two stages of denaturation of myosin follows from our results more or less directly; it leads to the further suggestion of two systems of bonds which maintain the native structural particles of lyosin. The destruction of the first system of bonds ($\Delta E \approx 53$ kcal/mole) is irreversible; it produces a decrease in stability of the protein in solution and evidently splits out large protein fragments. At the pH of maximum stability of ATP-ase, its active centers are further stabilized by bonds whose rupture requires the energy of about 85 kcal/mole. According to the results of Éidus and co-workers [16] the ATP-ase of myosin in the pH range of 5.8-6.2 passes reversibly into a state character-

TABLE 3. Rate Constants and Energies of Activation for Thermal Inactivation and Decrease in Stability of Myosin in Solution

pH buffer	ΔE_{oc}, kcal/m	ΔE_a, kcal/m	Q_{p_0}	Temp.	K_{oc}, $\log \frac{c}{c_0} \cdot \frac{1}{t}$	K_a, $\log \frac{Q_p}{Q_{p_0}} \cdot \frac{1}{t}$	$\frac{K_{oc}}{K_a}$
8,6, Bicarbonate	—	—	~3000	37	0,0440	0,0190	2,31
7,8, Borate	53 ± 5	85 ± 7	3750	35	0,01168	0,0021	5,58
» »			3750	37	0,0210	0,0044	4,77
			3500	39	0,0346	0,0126	2,75
6,1, Acetate	41 ± 7	47 ± 7	2780	29	0,00594	0,00369	1,61
			3310	32	0,0109	0,0059	1,85
» »			2680	34	0,0181	0,0130	1,40

Note: Solutions II, III, and IV – myosin concentration 3 mg/ml; K_{0c} and K_a – rate constants of decrease of stability in solution and decrease of ATP-ase activity; c_0 and c – concentration of native protein (initial and during heating) Q_{p_0} – initial Q_p; t – time from start of heating in minutes.

TABLE 4. Reversible and Irreversible Inactivation of Myosin and Decrease in Its Stability in Solution under the Influence of Cadmium Chloride (Solution II, myosin concentration 3 mg/ml. Native protein in % of protein precipitated in 0.22 % KCl after complete thermal inactivation)

Expt. no.	Q_p	$CdCl_2$ concentration, mg/ml	Activity, $\%Q_p$	Activity after reprecipitation by water (1 : 12) $\%Q_p$	Native protein, %
1	3360	0,010	86,5	98,0	97,5
		0,025	43,5	55,0	59,5
		0,050	2,0	14,4	13,0
2	3190	0,015	92,0	100,0	93,5
		0,025	36,0	31,0	34,0
		0,030	5,5	21,6	31,0
3	3350	0,015	89,5	—	100,0
		0,025	47,8	57,5	75,0
		0,030	25,4	62,0	59,5
4	4400	0,015	54,0	—	83,0
		0,025	19,9	38,1	67,5
		0,030	7,2	17,5	57,5
5	3710	0,015	72,0	—	86,0
		0,025	40,0	—	67,0
		0,030	30,0	—	68,0

ized by a value for ΔE of thermal inactivation of about 55 kcal/mole. From this we suggest that at pH 5.8-6.2 the active centers of ATP-ase are stabilized only by the first system of bonds. In such a case the loss of activity should occur at the same time and in parallel with the loss of stability in solution, without a "lag", as the sole process. Our experiments at pH 6.1 confirmed this suggestion (Fig. 5); it is true that as a result of a rough estimate we get here a somewhat lower value for ΔE.

Can we conclude from this a change in the structure of the protein with decrease in pH? The reversible rupture of covalent bonds with ΔE on the order of 80 kcal/mole is little likely; rather we can believe in the dissociation of numerous salt-like bonds in the ATP-ase part of the molecule, corresponding to H-meromyosin. From this point of view, the thermal stability of the active centers of the ATP-ase in myosin should be raised compared to H-meromyosin due to bonds which assure the integrity of the myosin particles.

Our preliminary experiments with cadmium chloride can be explained by assuming a double type of reaction with $CdCl_2$ of the protein. At small concentrations of Cd^{++} there is preferential binding of the SH groups of the ATP-ase active part of the molecule, which does not cause its denaturation.

This specific inactivation is reversible: Cd^{++} can be washed out by reprecipitation [3]. At higher cadmium concentrations, besides the specific binding, there is denaturation of the myosin which probably destroys the first system of bonds. This action at once leads to irreversible loss of ATP-ase activity, as would be expected on the basis of our experiments with thermal denaturation of myosin. The reversibility of the inactivation by cadmium depends on keeping the first system of bonds.

Analogous to this, under the influence of heat, destruction of only one of the myosin structures lower the thermal stability of its ATP-ase but does not cause loss of activity; for thermal inactivation there must be destruction of both systems of bonds. The assumption of two structures in myosin still requires more direct evidence, however.

SUMMARY

We have studied the kinetics of decrease in ATP-ase activity of myosin and its stability in solution under the action of raised temperature at pH 6.1 and pH 7.5-8.6. In the range of greatest thermal stability of ATP-ase (pH 7.5-8.6) both changes are connected with two different, successive reactions of the first order. The first reaction, which results in decreased stability of myosin in solution, and apparently in splitting of its particles, is characterized by an activation energy $\Delta E = 53 \pm 5$ kcal/mole. The second reaction, which results in inactivation of ATP-ase, occurs with a marked lag and a lower rate and requires $\Delta E = 85 \pm 7$ kcal/mole. At pH 6.1 both changes occur simultaneously as if due to one process with $\Delta E \cong 41$-47 kcal/mole.

Under the influence of cadmium chloride there are simultaneous reversible inactivation of part of the myosin and a slower irreversible inactivation which, differing from the reversible change, is related to decreased stability in solution.

The presence of two successive stages of thermal denaturation of myosin leads to the suggestion of two systems of bonds which maintain its native structure. The first system of bonds assures the native properties of the particles as a whole, while the second in a supplementary fashion stabilizes the active centers of the ATP-ase of myosin in the pH range of its greatest stability. In this range there must be destruction of the general and local structure of the protein for inactivation of the ATP-ase.

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THE STRUCTURE OF THE LIPOPROTEIN OF THE GRAY MATTER OF ANIMAL BRAINS

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In a previous study, one of us together with Ratnitskaya [1] showed that in the gray matter of beef brain most of the cholesterol and phosphatides were combined in a complex which dissolved in 0.25% NaOH solution. It was shown that this was a stable lipoprotein: It precipitated when the alkaline solution was acidified and dissolved again in 0.25% NaOH solution, retaining during this not only its lipoprotein composition, but also the original viscosity of the solution.

The investigation was continued and in the present paper we give some results on the structure and properties of this lipoprotein.

EXPERIMENTAL

In the lipoprotein which we studied the protein consisted of nucleoprotein and phosphorprotein. Therefore we felt it necessary first of all to show how the lipids were distributed in this protein between the nucleic acids and the protein. For this purpose we studied the action on the lipoprotein of NaOH in the presence of NaCl.

Action of NaOH on the lipoprotein in the presence of NaCl. The gray matter of beef brain was treated as described earlier [1] with this difference, that the ground brain tissue was treated five times with 7% solution of NaCl. For the investigation we used a solution of the treated tissue in 0.25% NaOH solution. To 50 ml of the resulting lipoprotein solution we added with stirring 2.6 ml of 1 N NaOH and then 15 g of NaCl. The mixture was stirred to full solution of the salt (the pH of this solution was about 10.7), after which it stood at room temperature until the next day. Then the mixture was acidified with HCl to a weakly acid reaction and centrifuged for 30 min at 2500 rpm. The clear solution was poured through a filter and its protein content was determined (the biuret reaction was always negative),* the precipitate was washed with a small amount of acidified water and dissolved in 50 ml of 0.25% NaOH solution. The resulting solution was centrifuged (usually the precipitate was slight) and the content in this solution of cholesterol and lipid and total phosphorus was determined. For the lipid determination the solution was extracted by the Bloor method with a mixture of alcohol and ether. Cholesterol was determined by the Bloor method, lipid phosphorus after distillation of the solvent and then mineralization, by the Fiske Subbarow method in the Braunshtein modification. Total phosphorus was determined in the alkaline solution of lipoproteins by the same method. The results of the experiments are given in Table 1.

When NaOH in the presence of NaCl acts on the lipoprotein for 24 hours (Table 1) nucleic acid is fully split from the nuceloprotein, while 87-95% of the total amount of cholesterol and 18-26% of the phosphates remain bound with the protein which is completely transferred to a 0.25% NaOH solution, since the supernatant liquid does not give a biuret reaction. Hence, this quantity of cholesterol and phosphatides were bound to protein and not to nucleic acid in the original lipoprotein. It is difficult to say how the remaining 5-13% of the cholesterol and 74-82% of the phosphatides were bound in this lipoprotein. It is possible that they were bound to the protein but were split from it under the action of the solvent used.

TABLE 1. Action of NaOH in the Presence of NaCl on the Chemical Composition of Lipoproteins (content of components in mg % fresh tissue)

Starting lipoprotein			Treated with NaOH + NaCl		
cholesterol	lipid P	total P	cholesterol	lipid P	total P
460	100	248	400	26	26
660	124	276	630	23	23
480	120	220	440	32	32

The question arises, how are the cholesterol and phosphatides bound to the protein in this newly formed complex. It has been suggested [2] that in lipoproteins cholesterol is bound with protein through phosphatides. This is indicated by the fact that when the water solution of the lipoprotein is extracted with ether in the presence of soap, cholesterol splits off but phosphatides remain bound with the protein. In order to answer this question with reference to our lipoprotein, we studied the ether extraction of lipids from the solution of this lipoprotein and also the extraction of cholesterol in the presence of Na oleate and Na glycocholate.

The experiments were carried out with the following samples: 15 ml of solution of lipoprotein; 15 ml of solution of lipoprotein + 2 ml of 0.01 M Na oleate; 15 ml of solution of

* In this centrufugate the reaction for RNA was strongly positive, and that for DNA negative.

TABLE 2. Content of Cholesterol and Lipid Phosphorus in the Protein-Lipid Complex after Its Extraction with Ether (complex obtained by action of NaOH and NaCl on original lipoprotein; content given in mg % of fresh tissue)

Before extraction		After extraction					
		by ether		by ether + Na oleate		by ether + Na glycocholate	
I	II	I	II	I	II	I	II
630	23	200	22	Trace	22	Trace	22
455	37,5	223	37	69	37	60	30
540	46	298	39	Trace	40	Trace	39
440	32	240	33	67	33	49	33

Designations: I) Cholesterol, II) lipid phosphorus.

TABLE 3. Content of Cholesterol and Lipid Phosphorus in the Lipoprotein after the Action of It of HCl (in mg % of fresh tissue)

Original protein		Lipoprotein treated with HCl			
		1 hour		24 hours	
cholesterol	lipid P	cholesterol	lipid P	cholesterol	lipid P
460	160	360	65	330	66
660	124	540	46	522	50
520	80	389	60	390	60

TABLE 4. Change in Chemical Composition of Lipoprotein after Its Extraction and Later Action of HCl (content of components in mg % of fresh tissue)

Original lipoprotein			Action of HCl on lipoprotein					
			without shaking			after shaking with ether		
I	II	III	I	II	III	I	II	III
480	100	4,2	375	80	3,7	270	39	1,8
480	102	4,0	378	75	3,5	270	40	Trace

Designations: I) Cholesterol; II) lipid phosphorus; III) nucleic acid phosphorus.

lipoprotein + 2 ml of 0.01 M Na glycocholate. All three samples were shaken for one half hour on a shaking machine and then the contents were transferred to a closed cylinder and allowed to stand at room temperature. On the next day the ether solution was drawn off and the content of cholesterol and lipid phosphorus in the alkaline solution was determined. The results of this experiment are given in Table 2.

From these results we see that with ether extraction in the presence of Na oleate and Na glycocholate, the cholesterol is removed almost completely (85-100%), while the phosphatides are not removed at all. Hence, we can consider that cholesterol in this complex is bound with the protein through phosphatides, providing the assumptions in the literature are correct. However, the strengths of the cholesterol bonds in this complex are not the same: In the presence of Na oleate or Na glycocholate ether removes 85-100% of the cholesterol, while without these substances under the same conditions ether removes only 40-65%. From this point of view, the results of studying the action of HCl on the original lipoprotein were interesting.

Action of HCl on the original lipoprotein. To 45 ml of the original solution of lipoprotein was added with stirring 5 ml of 1 N HCl (the pH of this solution was usually about 1,8-2,2). After an hour, the mixture was centrifuged, the opalescent supernatant liquid was poured from the precipitate and neutralized to a weakly acid solution; 3 g of NaCl was added to it and the resulting precipitate was again separated by centrifuging and was dissolved in 45 ml of 0.25% NaOH solution. The content of cholesterol and lipid phosphorus was determined in the solution. At the same time the original solution was treated in the same way but the mixture remained with the HCl until the next day, after which it was treated and analyzed as in the preceding case. The results of this experiment are given in Table 3.

The data in Table 3 show that HCl during an hour splits 19-25% of the cholesterol and 25-60% of the phosphatides from the lipoprotein, and after 24 hours the amount of cholesterol split is 21-28% and of phosphatides 25-60%, that is, the splitting of lipids from the lipoprotein essentially occurs in the first hour and further action of hydrochloric acid has no important effect. In an attempt to explain the biological significance of the lipoprotein under investigation, we have suggested that formation of lipoprotein increases the stability of the nucleoprotein in this complex. In order to test the correctness of this suggestion, we studied the stability of nucleoprotein toward HCl in this complex and the stability of the same nucleoprotein after splitting part of the lipoprotein from it by ether.

The experiments were carried out at follows: The initial solution of lipoprotein in the amount of 40 ml was shaken on a shaking machine with 160 ml of ether for 30 min; then the ether solution was poured off and again the same amount of ether was added and the mixture was again shaken for 30 min. This extraction was carried out three times, after which the mixture was transferred to a covered glass cylinder and allowed to stand until the next day. The ether solution was drawn off, and the water was used for the study. To 30 ml of each solution and also to 30 ml of the original solution we added 3.3 ml of 1 N HCl stirred, and after 15 min added NaOH to a weak acid reaction and 25 g NaCl; the precipitate was filtered off by centrifuging and again dissolved in 30 ml of 0.025% NaOH solution. In the resulting solution we determined the content of cholesterol, lipid phosphorus, and nucleic acid phosphorus by the method of Ivanova and Pravdina [3]. The results of the experiment are given in Table 4.

As the data show (Table 4) shaking the lipoprotein solution with ether, which splits off part off part of the lipid, decreases the stability of the nucleoprotein in this lipoprotein – when hydrochloric acid acts after shaking with ether, 43% of the nucleic acid is retained, while when hydrochloric acid alone acts on the lipoprotein, 78% of the nucleic acid is retained. Thus, the suggestion of the role of lipid in raising the stability of the nucleoprotein in this complex is fully confirmed.

SUMMARY

Investigation of the lipoprotein obtained from the gray matter of beef brain, in which the protein is composed of nucleoproteins and phosphoproteins, has been carried out. It has been shown that in this lipoprotein 87-95% of the cholesterol and 18-26% of the phosphatides are bound to the protein and not to the nucleic acid; it has been shown that this cholesterol is bound with the protein through the phosphatide. Cholesterol and phosphosphatide are not bound equally strongly in the lipoprotein; NaOH splits 5-13% of the cholesterol and 74-82% of the phosphatides; HCl 21-28% of the cholesterol, and 25-60% of the phsophatide. It has been shown that in this lipoprotein the lipids render the nucleoprotein more resistant to the action of hydrochloric acid.

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A STUDY OF TERMINAL AND MIDDLE AMINO GROUPS OF NATIVE AND DENATURED HUMAN SERUM ALBUMIN

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Study of the process of aggregation of human serum albumin after heat denaturation has established that the average molecular weight of albumin increases with increase in temperature, time of heating, and concentration of the protein in solution.*

Apparently, in the process of heat denaturation the protein molecule undergoes structural chemical changes which have still been little studied. A study of the character of thes changes is very important both for an understanding of the denaturation process itself, and for an analysis of the kinetics of association of the denatured molecule.

The amino acid composition of albumin is well known: The molecule of human serum albumin contains a large quantity of dicarboxylic acids (75 glutamic residues, 48 aspartic residues, 55 of lysine, 17 of cystine, and only one cysteine residue). These results indicate that the main bonds in the albumin molecule are peptide and sulfide links. Evidently aggregation of the protein also occurs because of them.

In 1950 the work of Iensen et al., [1] was published in which a number of sufficiently consistent experiments showed that free amino groups did not take part in the denaturation process. However, in 1955, Loiseleur and Catinot [2] in a study of the number of free functional groups in the process of denaturation of horse serum at 42-67° in sealed ampoules showed that the denaturation process was accompanied by a sharp change in the number of free NH_2 and COOH groups. The authors suggested that in irreversible denaturation there occurred the reaction $-COOH + -NH_2 \rightleftharpoons -CONH-$, which resulted in the formation of new peptide bonds.

As a test for the possibility of aggregation from free $-NH_2$ groups it appeared interesting to study the denaturation of the protein by the method of the dinitrophenyl derivatives [3] which permits determination separately of the quantity of amino groups at the end of the protein molecules and the number of amino groups gound in the middle of the chain.

The first work on determination of N-terminal groups of albumin was carried out in 1951: The method of dinitrophenyl derivatives was used for the determination of one N-terminal group, aspartic acid. Later, by the phenylisothiocyanate method, several N-terminal groups were found in albumin, of which two belonged to aspartic acid, one to methionine, one to histidine, and several groups to alanine. In 1954, Thompson [4] determined by the phenylisothiocyanate method as the N-terminal group aspartic acid and accompanying it, alanine. In 1955, Brown [5], discussing the above results, showed that in the albumin molecule with molecular weight 69,000 as N-terminal amino acids there was one molecule of aspartic acid and 57 free amino groups belonging to lysine.

However, the work of Reichmann [6] soon appeared which showed that the human albumin molecule consisted of two submolecules, each with a molecular weight of 32,000. If these results are accepted as true, then the molecule should have not one, but two N-terminal amino acids, and, correspondingly, two C-terminal acids. At the same time it was shown that in human albumin there is only one C-terminal group, leucine [7]. In 1956, Titani et al., [8] observed in albumin a new N-terminal group, cysteine.

However, Thompson in 1958 [9] in a review of all the above showed that cysteine is not present in the albumin molecule in the form of an N-terminal amino acid and only one N-terminal group appears in it, aspartic acid.†

The contradictory data on the N-terminal groups of albumin, obtained by the same method of the dinitrophenyl derivatives [8, 9], is evidently explained to some extent by the fact that in the hydrolysis of the DNP-protein along with the DNP-derivative of the end amino acid, soluble in ether, there are always formed dinitrophenol (DNP) and dinitroaniline (DNA) which also pass into the ether during ether treatment of the hydrolyzate

* Determination of the molecular weight of native and denatured albumin was carried out by L. V. Shchagina and S. E. Tukachinskii by the method of light scattering in a polarization nephelometer in the Biophysics Laboratory of the Leningrad Scientific Research Institute for Blood Transfusion.

† In 1959 appeared the work of G. Biserti, Biochim. et Biophys. Acta 34, 2 (1959) and B. Jirgensons and T. Ykenaka, Macromol. Chem. 31, 112 (1959), which confirmed that the N-terminal amino acid of human serum albumin was aspartic acid in the amount of 1 mole/g protein.

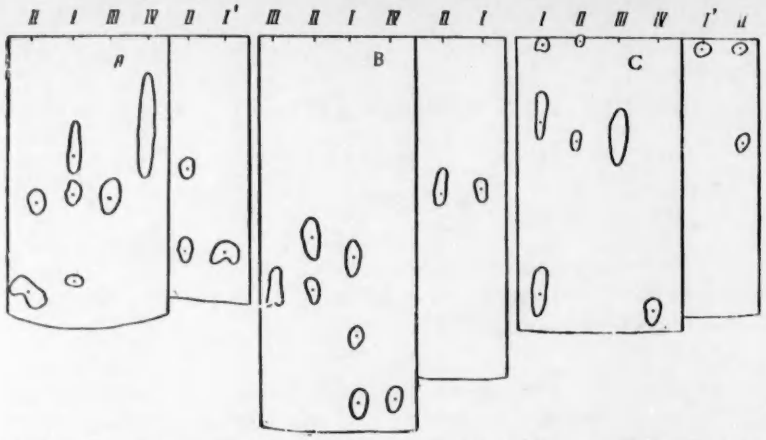

Fig. 1. Chromatogram of the ether fraction of the hydrolyzate from DNP-albumin. Solvents: A – Sodium phosphate, pH 6.2; B – phenol; C – toluene mixture. I – DNP-albumin; I' – DNP-albumin extracted from a spot; II – DNP-aspartic acid; III – DNP; IV – DNA.

of the DNP-protein and make the chromatographic analysis difficult. DNP evidently appears in the partial decomposition of DNP-amino acid during acid hydrolysis of the DNP-protein. DNA is also formed in the hydrolysis of DNP-protein, apparently because of the splitting of N-DNP-histidine.

Thus, the problem of the present study was to carry out a qualitative and quantitative analysis of the terminal and middle amino groups of native and denatured human serum albumin at different stages of aggregation and to develop accurate methods of chromatographic analysis of free amino groups.

EXPERIMENTAL

Determination of N-Terminal Groups of Human Blood Albumin Starting Substances

Protein. The work was carried out with electrophoretically pure human serum albumin which was denatured in the ultrathermostat, guaranteeing a constant temperature within ± 0.05° at 64° for 0.5, 2.5, and 3.5 hours. The molecular weight of the native albumin was determined by the light scattering method and equaled 72,000 which agrees well with the results of other authors.

Dinitrophenol (DNP) in the form of the sodium salt was obtained by treating acetamide (m. p. 114°) with fluorodinitrobenzene under the conditions described by Sanger for obtaining DNP-amino acids [3]. Along with the expected DNP amide of acetic acid at the end of the reaction there precipitated a clear yellow substance which by acidification was transformed into a scarcely yellowish material and after recrystallization from alcohol this melted at 70°. A mixed melting point with known DNP gave no depression. The elementary composition was verified by a Dumas analysis for nitrogen.

Sample I – 3.69 mg; obtained 0.514 ml. Weight of 1 ml N_2 = 1.1079 (24°, 735 mm)

Sample II – 4.52 mg; obtained 0.615 ml. Weight of 1 ml N_2 = 1.1405 (22°, 749 mm)

Percent N found – 15.4, 15.3; percent N calculated – 15.35.

Dinitroaniline (DNA) was produced by the Rubezbansk Works and after crystallization from alcohol had m. p. 182°.

Fluorodinitrobenzene (FDNB) was obtained by nitrating fluorobenzene and was purified by vacuum distillation.

DNP-albumin and DNP-aspartic acid were obtained by the usual process [3]. Then DNP-albumin was submitted to acid hydrolysis. After the excess hydrochloric acid had been removed, the product of hydrolysis was extracted from the water solution with ether.

Chromatographic Analysis

Qualitative determination of N-terminal groups of albumin. The ether soluble DNP-amino acids were submitted to chromatographic analysis in three different types of solvents: an aqueous solution of sodium phosphate (pH 6.2), phenol saturated with water, and a toluene mixture (toluene-pyridine-ethylene chlorohydrin-water in the ratio 1:1:0.1:0.1 by volume).‡

The results are shown in Table 1 and presented in Fig. 1. As Fig. 1 shows, the spots of the hydrolyzate of DNP-albumin during chromatography are always distributed into three areas: the spots of DNA, DNP, and DNP-aspartic acid.

Table 1 shows that when aqueous sodium phosphate is used as the solvent, the third spot of the hydrolyzate of DNP-albumin is always shifted more slowly than is the standard DNP-aspartic acid. The difference between the R_f of the third spot of the hydrolyzate and the R_f of

‡ In this work we used chromatography paper from the Volodarskii Factory in Leningrad.

TABLE 1. R_f Values of Ether Soluble DNP Derivatives

Length of front, mm	DNP-albumin			DNP-aspartic acid		DNP	DNA	Extracted 3rd spot of DNP-albumin
	I	II	III	I	II			
Sodium phosphate, pH 6.4								
140—148	Spread spot 0,3—0,4	0,52	0,82	0,58	0,85	0,58	Spread spot 0,3—0,4	
174—180		0,53	0,81	0,57	0,85	0,59		
152—154		0,54	0,82	0,53	0,84	0,56		
98—100		0,52	0,82	0,58	0,82	0,56		
146—153		0,54	0,84	0,55	0,86	0,59		
152—154		0,54	0,81	0,58	0,89	0,55		
		0,54	0,84	0,50	0,81	0,52		0,804
				0,51	0,805	0,54		
Average		0,53	0,82	0,56	0,85	0,57		
Phenol, saturated with water								
206—208	0,56	0,76	0,93	0,51	0,64	0,64		
160—165	0,53	0,72	0,93	0,44	0,60	0,64	0,91	
138—140	0,43	0,76	—	0,35	0,53	0,68	0,92	
172—174	0,42	—	—	0,35	0,53	0,67	0,93	
186—190	0,45	0,77	0,90	0,36	0,56	0,67	0,91	
175—180				0,46	0,63		0,90 0,94	0,45
Average	0,48	0,75	0,92	0,40	0,57	0,66	0,91	
Toluene mixture								
164—169	0,018	0,29	0,88	0,006	0,29	0,35		
154—156	0,026	0,29	0,87	0,007	0,35	0,33	0,93	
155—159	0,051	0,44	0,91	0,013		0,42	0,95	
136—138	0,044	0,35	0,95	0,014	0,35	0,37	0,97	
232—234	0,052	0,40	0,97	0,006	0,36	0,34	—	
117—119				0,033	0,44	0,46	0,88	0,033
146—147				0,034	0,37	0,41		0,034
Average	0,038	0,35	0,91	0,013	0,36	0,38	0,93	

Note: The values given in squares are the R_f values shown in Fig. 1.

DNP-aspartic acid is from 1 to 6%. The standard, DNP-aspartic acid, is separated into two spots itself; the first spot (R_f 0.53) is free DNP (R_f 0.575); the second is the true DNP-aspartic acid (R_f 0.82). In aqueous phenol the difference between the R_f values for the first spot of the DNP-albumin hydrolyzate and the R_f of the DNP-aspartic acids is more marked and always the first spot of the DNP-albumin hydrolyzate moved on the average faster than the standard spot.

In the chromatography of the toluene mixture, since the DNP-aspartic acid moved very slowly in this solvent and its path was short (from 1 to 6 mm), the difference between the R values was very great (60-90%).

Thus, chromatographic analysis of the ether layer of the hydrolyzate from DNP-protein which contains along with the terminal DNP-amino acid, DNP and DNA, carried out on a relatively short chromatogram, results in great variations in the R_f values.

Identification of DNP-aspartic acid. In order to test whether actually the spot of the DNP-albumin hydrolyzate which occurred closest to the spot of DNP-aspartic acid was DNP-aspartic acid, we cut the spot from the chromatograms obtained by using sodium phosphate (10 chromatograms), aqueous phenol (9 chromatograms), and toluene mixture (7 chromatograms). The 26 cut out spots were extracted three times with 5 ml of 0.1% $NaHCO_3$ solution and the main part of the resulting liquid was treated with ether. The ether was removed and the residue was treated with NH_3 according to Lowther [10]. For control the same treatment was given in parallel to DNP-aspartic acid. The resulting solutions, which contained the free amino acid, were carefully evaporated to remove excess NH_3, acidified with weak HCl, and submitted to chromatographic analysis. The chromatograms were treated with 0.2% ninhydrin dissolved in butyl alcohol.

As the standards we used the control solutions obtained by treating DNP-aspartic acid with NH_3 under the same conditions, DNP, DNP-aspartic acid, and aspartic acid.

The results, given in Fig. 2, show that with the NH_3 treatment, the product obtained by extraction of the third spot from the DNP-albumin hydrolyzate, which occurs close to the spot of DNP-aspartic acid when the chromatogram is sprayed with ninhydrin solution, is the lilac spot of an amino acid whose R_f (0.28) is close to the R_f of the lilac spot of the amino acid of the control solution (0.27) and the R_f of known aspartic acid (0.27).

TABLE 2. Determination of the Number of N-Terminal Groups

Average molecular weight of the protein	Weight of dry protein, mg	Ether extract, g	Solution deposited on chromatogram, ml	Extract of third spot of chromatogram, g (Solution A)	Average extinction of solution A	Concentration of DNP-aspartic acid in sample, mg	DNP-aspartic acid in sample, mg	Theoretical amount of DNP-aspartic acid in sample based on "monomeric" molecule, mg	Percent error with respect to theoretical value	
									without account of loss in hydrolysis	with account of loss in hydrolysis
					Native protein					
72,000	183	2.25	0.30	5.36	0.686	0.0141	0.567	0.764	25.8	10.8
72,000	186	1.00	0.09	5.05	0.505	0.0106	0.595	0.775	23.2	8.2
72,000	186	1.00	0.10	5.00	0.575	0.0120	0.600	0.775	22.6	7.6
					Denatured protein					
230,000	222	3.47	0.30	5.00	0.597	0.0124	0.720	0.925	22.1	7.1
230,000	222	3.47	0.40	5.59	0.770	0.0160	0.775	0.925	16.2	1.2
230,000	222	3.47	0.20	4.14	0.408	0.0086	0.618	0.925	33.1	18.1
900,000	224	4.00	0.20	5.26	0.307	0.0065	0.683	0.885	22.8	7.8
900,000	354	3.55	0.40	10.30	0.617	0.0128	1.17	1.470	20.4	5.4
450,000	300	1.00	0.10	8.70	0.551	0.0116	1.01	1.25	19.2	4.2
450,000	300	1.00	0.10	9.67	0.525	0.0110	0.958	1.25	22.0	7.0
					Hydrolyzed DNP-aspartic acid					
		3.09	0.20	28.39	0.700	0.148	6.56	7.64	14.0	
		3.09	0.20	75.00	0.264	0.056	6.34	7.64	16.0	

TABLE 3. Determination of Amount of Mono-DNP-Lysine in the Hydrolyzate from DNP-Protein

Average molecular weight of protein	Sample of dry protein, mg	Water extract, g	Solution deposited on chromatogram, ml	Extract from the chromatogram (solution A)	Average extinction of of solution A	Concentration of mono-DNP-lysine in 1 ml solution A, mg	Mono-DNP-lysine in sample, mg	Theoretical amount of mono-DNP-lysine in sample calc. on "monomeric" molec., mg	Percent error with respect to theoretical value
					Native protein				
72,000	183	10.0	0.1	14.68	1.22	0.0424	62.2	46.4	33.8
72,000	183	10.0	0.1	17.21	0.940	0.0324	55.8	46.4	20.0
					Denatured protein				
30,000	222	10.0	0.1	15.00	1.35	0.0428	64.4	56.4	11.4
30,000	222	10.0	0.1	15.00	1.42	0.0488	73.3	56.4	30.0
50,000	355	12.6	0.1	28.8	0.841	0.0290	105.3	90.0	17.0
50,000	355	12.6	0.1	28.8	0.858	0.0296	107.3	90.0	19.4

Repeated chromatography of the third spot from the DNP-albumin hydrolyzate. The lesser portion of the soda solution obtained by extraction of the yellow spot of the DNP-albumin hydrolyzate was concentrated and submitted to chromatography in different solvents. As column 6, Table 1, shows, the difference between the R_f of the spot obtained by extraction of the DNP-albumin hydrolyzate and the R_f of the spot of known DNP-aspartic acid lies within the limits of error of the experiment (0.1%).

Quantitative Determination of N-Terminal and Free Amino Groups in Native and Denatured Protein

Construction of calibration curve. We dissolved 0.01 g of DNP-aspartic acid in 5 ml of 0.1% $NaHCO_3$ solution. We placed 0.2 ml of this solution on chromatographic paper 35 × 40 cm. Chromatography was carried out with a water solution of sodium phosphate, pH 6, for 3-4 hours. The strip which corresponded to DNP-aspartic acid was cut out, extracted with a definite volume of 0.1% $NaHCO_3$ solution, and the intensity of the color of solutions of varying dilutions was measured on an SF-4 spectrophotometer (wave length 369 mμ). The results of five experiments were averaged and are shown in Fig. 3A and B.

Determination of the amount of DNP-aspartic acid destroyed in hydrolysis. To a solution containing 0.00764 g of DNP-aspartic acid was added 6 ml of 20% HCl and the mixture was heated in a sealed ampoule for 6 hours. At the end of the reaction, the contents of the ampoule were treated in the same way as was done after acid hydrolysis of DNP-albumin. A measured volume of 0.2 ml of the solution was chromatographed in a water solution of sodium phosphate, pH 6. The DNP-aspartic acid spot was cut out, extracted under the usual conditions, and the concentration of DNP-aspartic acid was measured on the SF-4 spectrophotometer. The data given in Table 2 (lines 11 and 12) show that the loss in the hydrolysis is 14-16%.

Determination of the amount of DNP-aspartic acid in the ether soluble fraction of the hydrolyzate from DNP-protein. We deposited 0.1 ml of soda solution of the ether soluble fraction, taken from 1 ml, on the chromatogram. After chromatography in a buffer solution, three yellow bands were clearly separated. The first corresponded to DNA, the second to DNP, the third to DNP-aspartic acid. The third band was extracted and the solution studied on the SF-4 spectrophotometer. The results, given in Table 2, show that the amount of terminal amino groups in albumin increase proportionally to the molecular weight of the denatured protein.

Determination of mono-DNP-lysine in the water soluble fraction of the DNP-protein hydrolyzate. The amount of mono-DNP-lysine in the water soluble fraction of the hydrolyzate of DNP-protein was determined in the same way as the DNP-aspartic acid in the ether soluble fraction of the DNP-protein hydrolyzate (calibration curve, Fig. 3B). The number of free amino groups belonging to lysine increases proportionally to the rise in molecular weight of the denatured protein (Table 3). The increased value of mono-DNP-lysine obtained from the experiment as compared to the theoretical value is explained by the fact that on the chromatogram water solution of mono-DNP-lysine is poorly separated from DNP and therefore the admixed DNP gives an increased extinction.

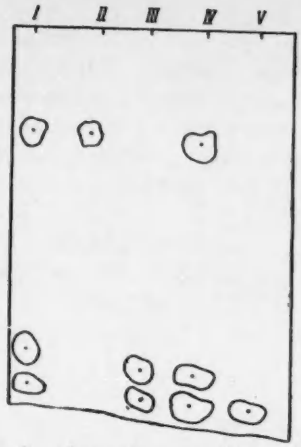

Fig. 2. Chromatogram of the soda extract of the SNP-albumin hydrolyzate spot after NH_3 treatment, sprayed with ninhydrin solution. Solvent: Butanol-acetic acid-water. I – DNP-aspartic acid treated with NH_3; II – aspartic acid; III – DNP-aspartic acid; IV – extract of DNP-albumin treated with NH_3; V – DNP.

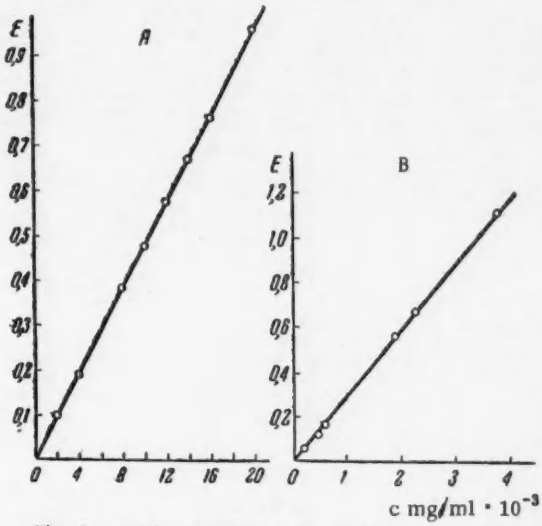

Fig. 3. Calibration curves for DNP-aspartic acid (A) and for mono-DNP-lysine (B).

SUMMARY

The N-terminal group of human serum albumin is aspartic acid in an amount of 1 mole per gram molecule of albumin with a molecular weight of 72,000. The number of terminal amino groups found in a "monomeric" molecule (mol. wt. 72,000) in native and denatured protein remains constant (equal to 1), independent of the degree of aggregation. The number of free amino groups belonging to lysine, calculated on the "monomeric" molecule, also remains constant, independent of the degree of aggregation.

The presence of dinitrophenol and dinitroaniline in the ether layer of the hydrolyzate of DNP-protein makes chromatographic analysis difficult, and for a final solution concerning the N-terminal groups of the protein we recommend separate extraction of the spots of the hydrolyzate from the DNP-protein and their repeated chromatographic analysis.

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A BACTERIAL METHOD FOR THE PREPARATION OF γ-AMINOBUTYRIC ACID

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Certain bacterial species are known to contain very active amino acid decarboxylases [1-3].

The organisms of the E. coli group characteristically contain glutamic decarboxylase. This enzyme is always found in these bacteria, irrespective of culture technique. The reaction product of glutamate decarboxylation is γ-aminobutyrate. This appeared to present a practical, feasible means for the preparation of γ-aminobutyrate whose physiological functions have been a subject of considerable interest [4].

The procedure offered below is relatively simple and may be carried out in any laboratory. The optimal decarboxylation of glutamate by E. coli cells proceeds at pH 4.5. This pH may be achieved with the substrate alone - glutamic acid hydrochloride. Since the decarboxylation proceeds without any marked shifts in pH, the use of buffers is obviated. We have demonstrated that under those conditions glutamic acid was almost completely decarboxylated by the bacterial suspension in aqueous solution.

METHODS

The following procedure was used.

Glutamate may be decarboxylated by any strain of E. coli. The decarboxylating activity of any strain should be checked prior to use, as described previously [5].

In order to obtain the necessary bacterial suspension, active strains were inoculated onto slants containing nutritive agar with 2% glucose. The cultures were harvested from the medium after 17-18 hours of growth at 37°. The cells were washed off the agar with physiological saline solution, centrifuged and washed twice with saline. The bacterial pellet was then suspended in an aqueous solution of glutamic acid. The final pH, which should be 4.5, is usually obtained with a 0.5% solution of glutamic acid hydrochloride. Bacterial cells obtained from 10 slants were suspended in 10 ml of this solution, the suspension was placed in a collodium dialysis sac fastened to a glass rod, and the bag was immersed in a flask containing 100 ml of the glutamic acid solution. The flask was incubated at 37°.

Samples of the reaction mixture were withdrawn at intervals for the monitoring of reaction progress. The samples were applied to paper and subjected to electrophoresis. The electrophoretogram of samples withdrawn after one, three, and six hours of incubation are shown on Fig. 1. The electrophoretogram (1) of the sample taken at one hour shows an intense band of glutamic acid and but a faint band corresponding to the newly formed γ-aminobutyrate. After three hours of incubation (2), the band corresponding to γ-aminobutyrate was very pronounced, while that corresponding to glutamate was faint. Finally, after six hours, the reaction mixture (3) contained practically exclusively γ-aminobutyric acid. Thus, under the conditions specified, practically complete decarboxylation of the added glutamate took place in six hours. This reaction was considerably faster if the cellular suspension was substituted by an enzymatic preparation of glutamic decarboxylase described in an earlier communication [6].

The solution in the outer flask was evaporated to dryness on the water bath, leaving a white crystalline powder. Paper chromatography of solutions of the solid showed, after development with ninhydrin, the appearance of only one ninhydrin-positive spot, corresponding to γ-aminobutyric acid.

In order to demonstrate that γ-aminobutyric acid, and not α-aminobutyric acid, was the product of the reaction, use was made of the ability of α-amino acids to form copper complexes. This reaction is utilized in the quantitative assay of amino acids [7]. The copper complexes of amino acids thus formed do not give the characteristic reaction with ninhydrin. On the other hand, γ-amino acids do not form complexes with copper and therefore react with ninhydrin after copper treatment [8].

The amino acid to be identified was applied to chromatographic paper in the usual manner. The paper was then dusted with finely powdered basic copper carbonate [$CuCO_3 \cdot Cu(OH)_2$] along the path to be traversed by the spot. The amount of dust applied should be such as to impart only a just perceptible color to the paper, since heavier dusting interferes with the ninhydrin reaction. The paper was then irrigated as usual with the solvent (butanol/acetic). The results are shown on Fig. 2.

The position of α-aminobutyric acid may be seen in strip 1. After copper treatment (strip 2) the spot disappeared. Strip 3 contained γ-aminobutyric acid treated with copper. After spraying with ninhydrin a yellow spot

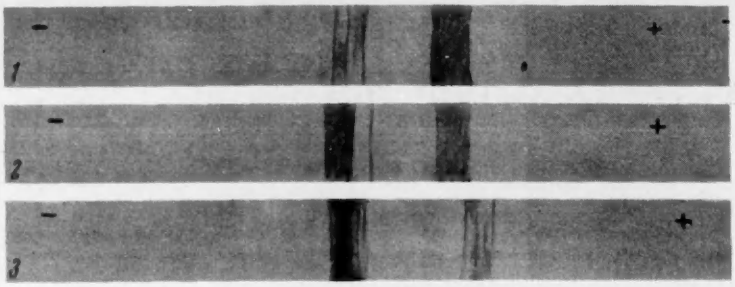

Fig. 1. Electrophoretic separation of samples withdrawn during the course of glutamic acid decarboxylation by bacterial suspension. 1) One hour sample; 2) three hours sample; 3) six hours sample.

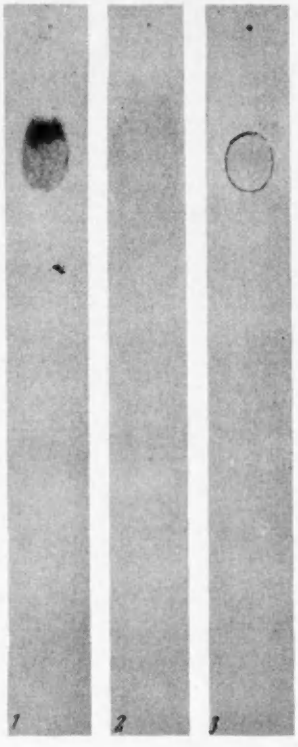

Fig. 2. Descending chromatography of α-aminobutyric and γ-aminobutyric acids. 1) Control (α-aminobutyric acid); 2) α-aminobutyric acid, copper-treated; 3) γ-aminobutyric acid, copper treated.

appeared, which is not seen very distinctly on the photograph. It should be noted that, in the solvent system employed, the R_f values of α-aminobutyric acid and of γ-aminobutyric were identical.

It was thus established that the decarboxylation of glutamic acid by a suspension of E. coli involves only the α-carboxyl and that the product is exclusively γ-aminobutyric acid.

SUMMARY

A simple method for the preparation of γ-aminobutyric acid from glutamic acid is described. The method involves the use of an E.coli cell suspension.

The product, γ-aminobutyric acid, was identified by paper chromatography using paper treated with basic copper carbonate.

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THE CHEMICAL COMPOSITION AND PHYSICOCHEMICAL PROPERTIES OF GONADOTROPIC HORMONES

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At the present time the interconnection has been established between the function of the sex glands of the organism and the gonadotropic hormones produced in the anterior lobe of the pituitary [1-5].

Studies in recent years have shown that the pituitary secretes three hormones of a protein nature which have gonadotropic activity: the follicle stimulating hormone, the luteinizing hormone or the hormone which stimulates development of interstitial tissues, and the luteotropic hormone which stimulates the development and maturation of the corpus luteum [27, 28]. In pregnancy, in addition to the pituitary, the placenta is a source of gonadotropic hormones, secreting the chorionic gonadotropin.

The role of the pituitary in the development of the sex glands was first demonstrated in 1927 by the work of Zondek [1]. However, the chemical nature of the gonadotropic hormones of the pituitary, the path of their synthesis and metabolism in the organism, and also the chemical nature of the end products of gonadotropic metabolism excreted in the urine have not yet been sufficiently investigated.

The gonadotropic hormones of the pituitary appear in the early stages of ontogenesis in humans and animals and are secreted until advanced old age, undergoing at different periods in the life of the organism quantitative and probably also qualitative changes [8, 9]. However, up to a definite age, that is, up to the period of sexual maturity, the sex glands of the growing organism do not react to the gonadotropic hormones secreted by the pituitary [10]. At the same time, injection into the immature animal of gonadotropic extracts from the urine of adult individuals or of chorionic gonadotropin from pregnancy urine causes easily observed stimulation of the gonads [1, 11]. The reason for this is not yet clear. It is not known whether it is connected only with the fact that the pituitary of the young organism produces gonadotropic hormones in such small amount that they are insufficient to stimulate the gonads, or whether the hormones are secreted during this period in a chemically inactive state.

Study of the path of synthesis, metabolism, and excretion of gonadotropic hormones during ontogenesis can be helpful for an understanding of the processes of normal formation and development of the female organism and can perhaps also disclose the reasons for some pathological conditons which lead to sterility. There are almost no data in the contemporary literature on the chemical composition and physicochemical properties of gonadotropic hormones in the urine of children. It is known only that the urine of children contains a very small amount of gonadotropins and there is information on the carbohydrate composition of the molecules of these hormones [12, 13].

The preparative isolation of gonadotropic hormones from the urine is sufficiently difficult since its gonadotropin content is low and special methods are required to concentrate these substances. At the present time many methods have been suggested for separation and purification of gonadotropic hormones from urine. They include precipitation by alcohol [1-14], by acetone [13, 14], by tannic acid [16], by ultrafiltration [15, 17], by adsorption on benzoic acid [15], permutite [19], or kaolin [14, 18].

For isolation of gonadotropic hormones from the urine of children we have used the method of adsorption on kaolin suggested by Dekanski [14] and improved and detailed by Loraine [18]. For comparison we used the method of adsorption on benzoic acid suggested by Katzman and Doisy for isolation of the chorionic gonadotropin [15].

EXPERIMENTAL

Materials studied: The source of gonadotropic hormones was the urine of normally developing girls of three age groups: 3-4 years, 5-6 years, and 6-7 years. There were 10-15 children in each age group. The urine was collected daily during the course of the day and was treated as received.

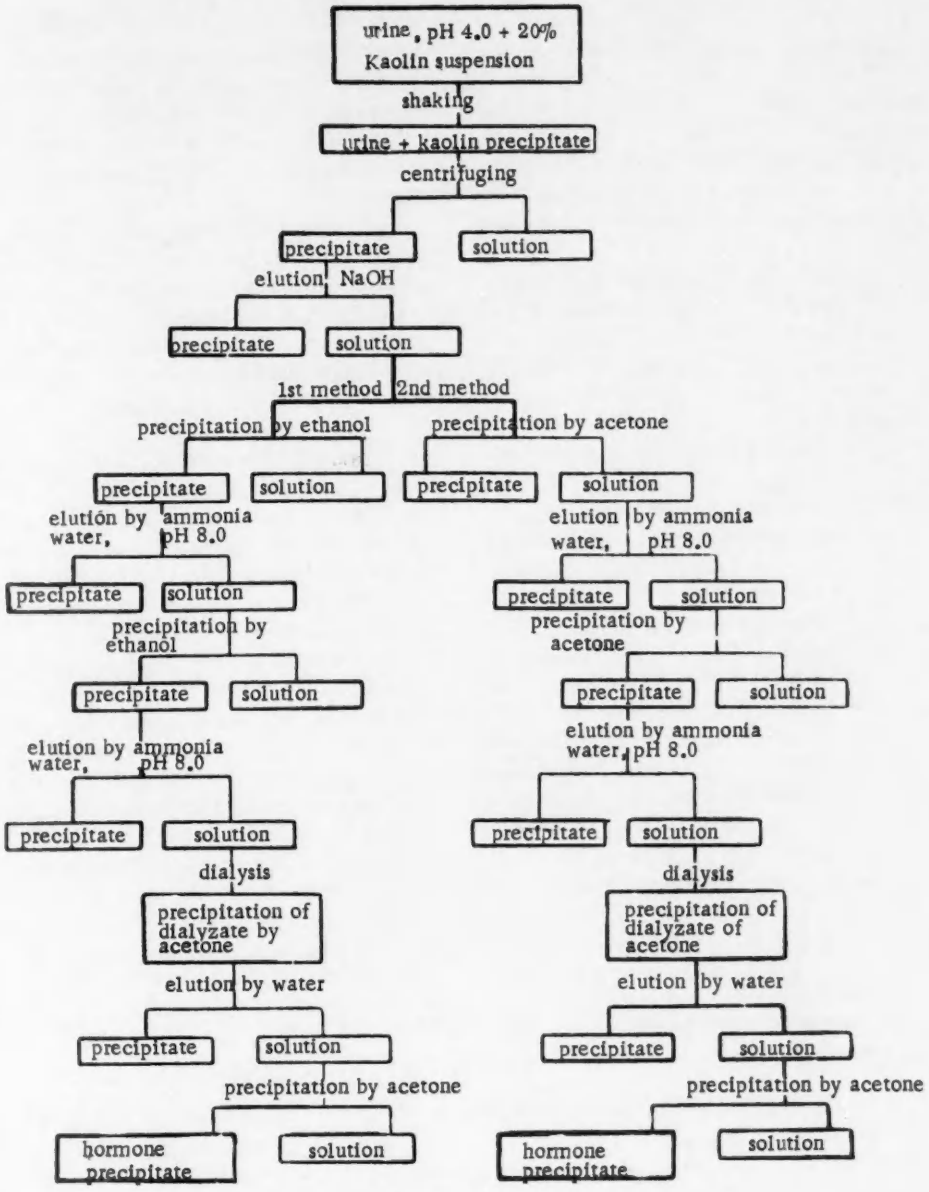
Method of Isolating Gonadotropin

Adsorption on kaolin. The method was basically that of Dekanski with some modification by us (scheme).

To a definite volume of urine, acidified with concentrated HCl to pH 4.0 was added a 20% suspension of kaolin in water calculated at 2.5 g of kaolin per ml of initial urine. The mixture was carefully stirred for an hour and remained in the refrigerator (at 2-4°). After it had settled, the supernatant liquid was poured off by decantation and a new portion of urine was added to the kaolin after analogous treatment. The procedure was repeated to obtain a kaolin precipitate from the treatment of 50 liters of urine (1.250 g of kaolin). The kaolin precipitate was separated by centrifuging and twice treated with 1 N and 0.5 N NaOH to obtain a solution with pH

Scheme of Isolation of Gonadotropic Hormones from Urine

by Adsorption on Kaolin



11.0. The alkaline eluate after the first and second treatments was separated by centrifuging and combined. The kaolin precipitate was discarded.

Further treatment of the alkaline eluate was carried out by two methods.

1. After acidification of the alkaline eluate with glacial CH_3COOH to pH 4.0, it was treated with triple the volume of 96% alcohol with cooling. The precipitate was separated and repeatedly treated with small portions of distilled water (pH 8.0-8.5 by addition of NH_4OH) to negative biuret reaction in the wash water. The insoluble precipitate was discarded. The combined water extracts were again treated with ethanol as described above, and the resulting precipitate was treated with water. The combined water extracts were dialyzed against cold distilled water. The hormone was twice precipitated from the dialyzate with five times the volume of cold acetone, pH 4.0-5.0. The resulting precipitate was separated, washed with cold acetone, and dried in the refrigerator.

2. The alkaline eluate, after acidification to pH 4.0-5.0, was treated with five times the volumes of cold acetone. All the later operations were carried out in the same order as in the first method, with only the difference that treatment with ethanol was replaced by treatment with acetone.

We used the second variant of treating the alkaline eluate because the use of ethanol led to incomplete precipitation of the protein. The use of acetone as the precipitant gave fuller precipitation. To test the effect of the method of treatment on the activity of the hormone we used the first variant of the method for working up a small amount of urine from women in the state of full development.

Adsorption on benzoic acid. To a definite volume of urine acidified to pH 4.0-4.5 by glacial CH_3COOH we added a saturated solution of benzoic acid in acetone calculated at 50 ml/liter of initial urine. After the solution with its precipitate had been shaken energetically for an hour, it stood until the next day in the refrigerator. The precipitate was separated and treated with acetone to dissolve the benzoic acid.

The insoluble precipitate which contained the gonadotropin was washed free of benzoic acid with acetone. The air dried precipitate was repeatedly eluted with small portions of 40% ethanol, pH 8.0 (addition of NH_4OH) to a negative biuret reaction in the eluate. The insoluble precipitate was discarded. To the combined eluates, acidified to pH 4.0-5.0 was added a triple volume of 96% ethanol with cooling. The precipitate was separated, washed with cold acetone, and ether, and again repeatedly treated with 40% ethanol, pH 8.0. The insoluble precipitate was discarded and the combined eluates, acidified to pH 4.0-5.0, were treated with triple the volume of cold acetone. The precipitate was separated, washed and dried with acetone and ether, dissolved in water, and submitted to dialysis against distilled water. The dialyzate, acidified to pH 4.0-5.0, was treated with five times the volume of cold acetone. The precipitate was separated, washed with acetone, and dried in the refrigerator.

The yield of the preparations by the various methods of isolation is given in Table 1.

BIOLOGICAL STUDY

The gonadotropic activity of the resulting preparations was studied by the method of Levin and Tyndale, based on the increase in weight of the uterus of immature mice [16, 20]. As the unit of gonadotropic activity by this method we used a 100% increase in weight of the uterus.

We used the following plan of biological study of the resulting preparations: each preparation was studied in a group of three 21 day old mice (12 animals in the control group). Each of the studied animals over a three day period received six subcutaneous injections of 0.3 ml of a freshly prepared solution of the sample in physiological saline. On the fourth day the animals were killed, the uterus was removed, separated from the fatty tissues, and weighed on a torsion balance. The control animals were studied in an analogous way.

The resulting data (Table 2) show that the isolated preparations have gonadotropic activity, most clearly expressed in the preparation from the urine of 3-4 year old girls. One mg of it converted to absolutely dry and ash-free substance causes an increase in weight of the uterus of the experimental mice which averages 137% of the controls. At the same time, 1 mg of the preparation from urine of 6-7 year old girls causes an increase in weight of the uterus of the experimental animals of 9-11%. The preparation from the urine of girls of the same age obtained by adsorption on benzoic acid was more active; 1 mg of it caused an increase in weight of the uterus of immature mice of 85%. However, gonadotropic preparations from the urine of children were less active than preparations from the urine of mature women. While 1 mg of the latter caused an increase in weight of

TABLE 1. Yield of Gonadotropin by Different Methods of Its Isolation

Preparation No.	Obtained from urine of	Method of treatment	Amount of urine treated, liters	Amount of substance obtained, g	Color
1	Girls 3-4 years	Adsorption on kaolin, Variant I	105	1.39	Light brown
2	Girls 5-6 years	The same	50	0.875	Brown
3	The same	The same, Variant II	50	0.240	Light gray
4	Girls 6-7 years	The same	35	3.202	Light gray
5	The same	The same	30	1.352	Dark brown
6	The same	Adsorption on benzoic acid	20	0.193	Gray
7	Fully grown women	Adsorption on kaolin Variant I	13.3	0.640	Gray

TABLE 2. Average Data for Results of Biological Study of Gonadotropic Preparations from Urine of Girls of Different Age groups

Preparation No.	Obtained from urine of	Weight of animals		Preparation of absolutely dry, ash-free substance, mg/animal	Average weight of uterus, mg	Average increase, in % of control	Increase produced by 1 mg of substance
		beginning of expt.	end of expt.				
1	Girls 3-4 years	8.2	8.5	2.5	11.3	343.0	137.2
2	Girls 5-6 years	8.6	9.2	1.81	6.3	192.0	105.0
3	Girls 5-6 years	9.1	9.5	3.02	8.0	242.0	80.1
4	Girls 6-7 years	8.0	8.6	16.30	6.0	182.0	11.1
5	Girls 6-7 years	8.0	8.8	15.2	4.7	142.0	9.3
6	Girls 6-7 years Adsorption on benzoic acid	8.9	9.5	3.06	8.7	262.0	85.6
7	Fully developed women	9.1	9.6	0.91	7.3	222.0	244.0
	Control animals	8.8	–	–	3.3	100.0	–

TABLE 3. Results of Chemical Study of Gonadotropic Hormone Preparations from the Urine of Girls of Different Age Groups

Preparation No.	From urine of	Ash, %	Moisture, %	Water and ash free substance, %	Total N, %	NH_3-N, %	Total N – NH_3-N, %	Protein N, %	Nonprotein N, %
1	Girls 3-4 years	17.22	13.22	69.51	10.39	2.33	8.6	8.4	0.2
2	Girls 5-6 years	31.44	15.86	50.30	11.05	Not determined			
3	The same	2.1	13.19	83.91	10.57	1.42	9.15	8.9	0.25
4	Girls 6-7 years	30.97	14.56	54.45	11.88	2.44	9.40	9.19	0.21
5	The same	31.10	18.01	50.89	14.67	4.18	10.49	10.22	0.21
6	The same, adsorbed on benzoic acid	3.94	11.28	84.78	11.50	Not determined			
7	Mature females	58.82	17.93	25.25	12.44	Not determined			

the uterus of immature mice of 244%, the most active preparation from the urine of children caused an increase of 137%.

CHEMICAL STUDIES

For a study of the total chemical composition of the preparations of gonadotropins we made the following determinations: moisture content, ash elements, total and protein nitrogen. The use of aqueous ammonia for removal of the gonadotropins made it impossible to determine ammonia bound as a salt in these preparations.

Method of investigation. The determination of moisture content and ash elements was carried out in the usual way. The content of total nitrogen was determined by mineralization of the preparations by heating with a mixture of concentrated H_2SO_4 and K_2SO_4 with later determination of the evolved ammonia by the method of Seligson [21]; protein nitrogen was determined by precipitation with sodium phosphotungstate in an acid medium, followed by mineralization of the protein precipitate by heating with the mineralizing mixture and determination of the evolved ammonia by the Seligson method. Free (salt) ammonia was determined in a water solution by the Seligson method. The results of the determinations are given in Table 3.

As the data in Table 3 show, the content of protein nitrogen in the preparations of gonadotropic hormones from the urine of children makes up 98% of the total content of nitrogenous substances (without ammonia). However, some preparations contain more ash elements.

PHYSICOCHEMICAL STUDIES

For a study of the physicochemical characteristics of the preparations of the gonadotropic hormones as substances of a protein nature we carried out electrophoretic investigations, studies in the ultracentrifuge, and studies of changes in the coefficient of diffusion.

Electrophoretic investigation. Electrophoresis was carried out on paper, using buffer systems of different chemical types and with different pH values (from 5.1

to 9.18). The concentration of the solutions studied corresponded to a 2% solution of the protein; the time of electrophoresis was varied from two to three hours, the voltage from 20 to 40 v/cm. The position of the hormones was determined by coloring the electrophoretogram with a mercuric acetate solution of bromphenol blue. The ionic strength of the veronal buffer was 0.042; in all the other buffer solutions it was 0.055. The results were as follows. In buffer systems with pH values in the acid region the hormones studied had no mobility in the electrical field and remained at the point of deposition as a clearly visible spot. In the electrophoretograms of all the preparations only one spot was observed. When the electrophoresis was carried out in an alkaline medium (pH 7.24-9.18), the preparations showed a very slight increase in mobility. Separation into different fractions did not occur here. The hormones appeared as an extended, diffuse spot (Fig. 1). Thus, all the isolated preparations are electrophoretically homogeneous proteins.

Study in the ultracentrifuge.* Determination of the sedimentation of the gonadotropin preparations was carried out in a Svedberg ultracentrifuge at a rotation rate of the rotors of 61,000 rpm. The preparations were dissolved in water, made alkaline to pH 8.6 by the addition of $NaHCO_3$. The sedimentation diagrams are given in Fig. 2. Analysis of the sedimentation curves shows that the gonadotropins isolated by the method of adsorption on kaolin behave as proteins with a rather low molecular weight, and an apparent heterogeneity due to the presence of high molecular weight proteins was not observed. However, we cannot exclude the possibility of

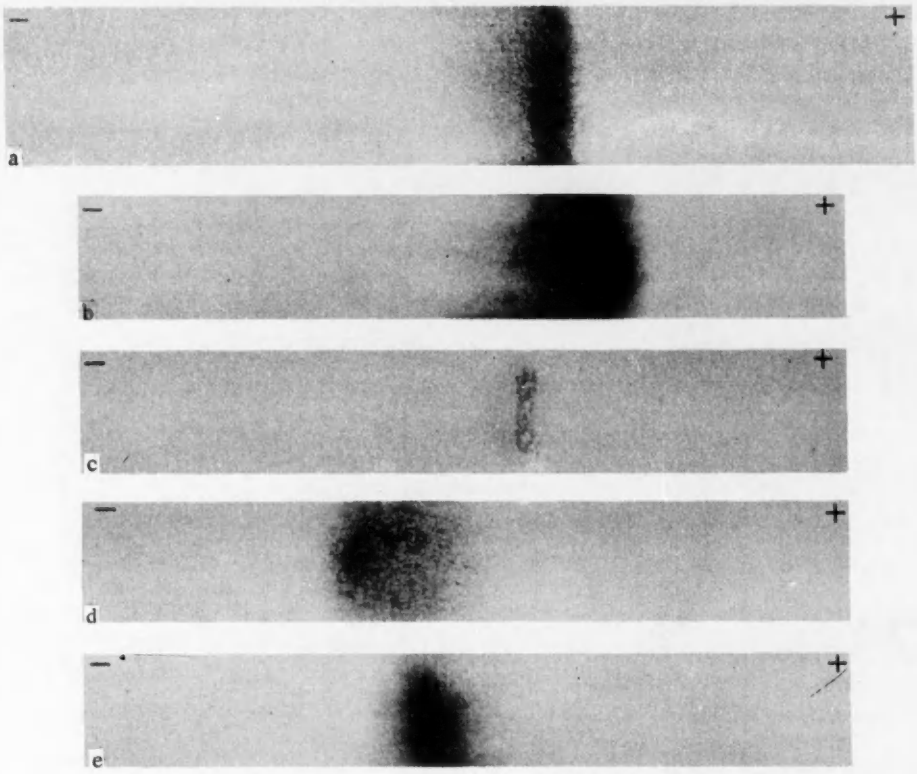


Fig. 1. Electrophoretograms of gonadotropin preparations. a) Adsorption on kaolin, Na veronal buffer (pH 8.6), μ = 0.042, 30 v/cm, 3 hours; b) adsorption on benzoic acid, Na veronal buffer (pH 8.6), μ = 0.042, 30 v/cm, 3 hours; c) adsorption on kaolin, acetate buffer (pH 5.1), μ = 0.55, 30 v/cm, 2 hours; d) adsorption on kaolin, phosphate buffer (pH 8.67), μ = 0.055, 20 v/cm, 3 hours; e) adsorption on kaolin, phosphate buffer (pH 9.18), μ = 0.055, 15 v/cm, 3 hours.

* The studies in the ultracentrifuge, like the measurements of coefficient of diffusion described below, were carried out in the Laboratory of Protein Biochemistry of the Institute of Biological and Medical Chemistry, Academy of Medical Sciences, USSR (Moscow) by Chief Scientific Co-worker V. O. Shpikiter, to whom the authors express their thanks.

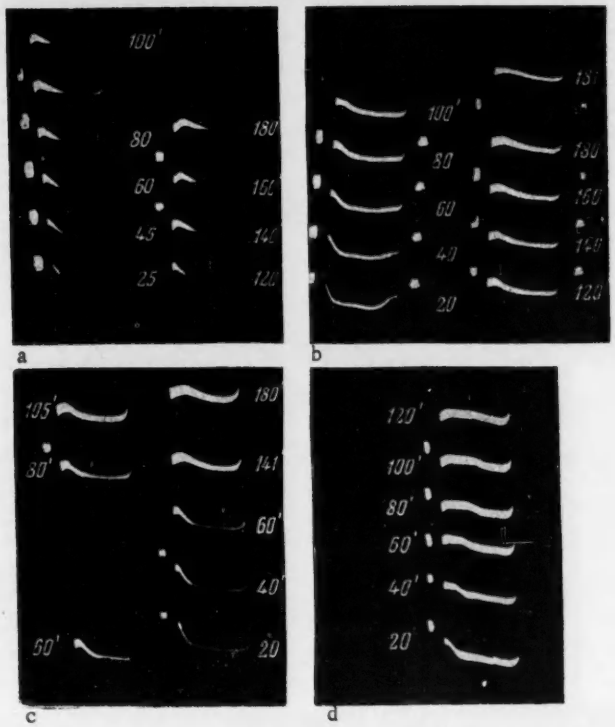


Fig. 2. Sedimentation of gonadotropin solutions in the ultracentrifuge. A) Preparation 3. Rate of rotation of rotors, 930 revolutions/sec, temp. 24°. The preparation settles slowly. The form of the peak is nearly symmetrical. No visible heterogeneous admixtures can be observed under the conditions of the experiment. B) preparation 4. Rate of rotation of rotors 1020 revolutions/sec, temp. 24°. Preparation settles slowly. Form of peak nearly symmetrical. No visible heterogeneity can be observed under conditions of the experiment. C) Preparation 5. Rate of rotation of rotors 1020 revolutions/sec, temp. 26°. Preparation settles slowly. Form of peak nearly symmetrical. No visible heterogeneity under experimental conditions. D) Preparation 6 (adsorbed on benzoic acid). Rate of rotation of rotors 1020 revolutions/sec, temp. 25°. When the preparation is sedimented in the ultraventrifuge we observe two diffuse peaks which indicate its nonhomogeneity.

some heterogeneity due to the presence of some sort of low molecular weight admixtures which are not observed under the conditions of the experiment. At the same time the preparation of gonadotropic hormone obtained from the urine of 6-7 year old girls by adsorption on benzoic acid is heterogeneous and shows the presence of at least two protein components.

Using these results we tried to calculate the sedimentation constants for the three preparations of gonadotropins which under the experimental conditions showed no visible signs of heterogeneity. The determination of the sedimentation constant was carried out by the formula:

$$S = \frac{x_2 - x_1}{\frac{x_2 + x_1}{2}\omega^2 (t_2 - t_1)},$$

where S is the sedimentation constant, x_1 and x_2 the distances from the middle of the sedimentation boundary ("peak") to the axis of rotation of the cell in the times t_1 and t_2, ω the angular velocity of rotation. The results are given in Table 4.

These results are only orienting and give only an idea of the approximate order of magnitude of the sedimentation constants of the gonadotropic hormones from the urine of children, isolated by adsorption on kaolin.

Determination of diffusion coefficients. Diffusion coefficients were determined for preparations No. 3 and No. 4. The determination was carried out in a Lamm apparatus. Photography of process of diffusion was carried out after 8 hours 25 min, 17 hours 50 min, and 22 hours 30 min from the beginning of the experiment. Diffusion

TABLE 4. Sedimentation Constants of Some Preparations of Gonadotropic Hormones

Preparation No.	From urine of	S
3	Girls 5-6 years	0.60×10^{-13}
4	Girls 6-7 years	0.78×10^{-13}
5	The same	0.78×10^{-13}

coefficients were calculated by the method of second moments and area and maximum ordinates [24]. The value of the coefficients for both preparations was on the order of 3.5×10^{-7} cm/sec. This value, like the values for the sedimentation constant, is only orienting, and requires further improvement.

DISCUSSION OF RESULTS

The electrophoretic study carried out over a wide enough range of pH values and in buffer systems of different chemical nature showed that the gonadotropin preparations isolated from the urine of children were proteins which migrated to the anode in an electrical field. The isoelectric point of these proteins obviously lies at a pH below 5.0. Thus, the electrophoretograms under the conditions studied show only one protein fraction. One protein fraction was also observed in electrophoresis in the Na veronal buffer at pH 8.6 and ionic strength of the buffer equal to 0.042, that is, under optimal conditions in which the gonadotropins have the greatest mobility and are easily separated from the other proteins of urine [22].

Present methods for preparative separation of proteins do not permit us to speak with sufficient assurance of the homogeneity of one or another isolated protein. Therefore our results permit us merely to say that by the use of paper electrophoresis with preparations of gonadotropic hormones from the urine of children we do not observe heterogeneity due to the presence of perceptible amount of other proteins.

Investigations with the ultracentrifuge showed that the preparations of gonadotropic hormones obtained by adsorption on kaolin do not contain high molecular weight admixtures and under the conditions of the experiment behave like homogeneous substances. However, the possibility of the presence of low molecular weight admixtures, which are not detected under the experimental conditions, is not excluded. At the same time, sedimentation in the ultracentrifuge indicated the presence of not less than two protein substances in the preparation obtained by adsorption on benzoic acid. We still do not know which of these proteins is connected with the relatively high gonadotropic activity of the preparation. The possibility is not exluded that one of the proteins in the preparation is a denaturation product of the hormone, formed during the processes of its isolation and purification.

For the gonadotropic hormones which in the ultracentrifuge do not show any signs of heterogeneity (preparations 3, 4, and 5) we have tried to calculate the molecular weight, using our data on the sedimentation constant (S) and the coefficient of diffusion (D). The molecular weight (M) of these hormones is calculated by the formula $M = SRT/D(1-V\rho)$, where R is the gas constant, T the absolute temperature, V the partial specific volume of the protein, ρ the density of the solvent, on the order 16,500-20,000. The relatively low molecular weight of the gonadotropin preparations is confirmed by their behavior in the ultracentrifuge, by their slow sedimentation.

Although the results on S, D, and molecular weight of the gonadotropic hormones isolated from the urine of children are very approximate, yet it is interesting to compare them with analogous results obtained for gonadotropins isolated directly from the pituitary. According to the data of Li [25], S for the pituitary follicle stimulating hormone is 4.7×10^{-13}, $D = 6.0 \times 10^{-7}$, and the molecular weight is 67,000. According to the results of Shedlovsky [26], luteinizing hormone from swine pituitary has $S = 5.39 \times 10^{-13}$, $D = 5.9 \times 10^{-7}$, and molecular weight 90,000.

The protein which we isolated from the urine of children, which shows gonadotropic activity, has a considerably lower molecular weight, that is, compared to the pituitary gonadotropin it is a lower molecular weight substance.

This suggests to us that high molecular weight proteins like the pituitary gonadotropic hormones are included in metabolic dynamic processes in the living organism, processes which occur in the gonads and lead to very considerable chemical transformations. The substances formed as a result of these transformations have a very low molecular weight and so can pass through the "filter" of the kidney and can be extracted in the urine. The end products of gonadotropic hormone metabolism formed in this way show biological activity, and this permits us to suggest the presence in pituitary gonadotropins of specific structures which are evidently only fragments of the molecule and with which the gonadotropic activity of these biologically active proteins is connected.

SUMMARY

The modified Dekanski method of adsorption on kaolin and also the method of adsorption on benzoic acid

permit isolation from the urine of normally developing girls (from three to seven years of age) of preparations which have gonadotropic activity. The resulting hormonal preparations are distinguished by a high content of protein nitrogen, about 98% of the total content of nitrogenous substances.

Investigation by the method of paper electrophoresis shows that the hormone preparations are proteins which migrate to the anode in the electrical field.

The optimum conditions for paper electrophoresis for these hormone preparations are: use of a sodium veronal buffer, pH 8.6, ionic strength 0.042, voltage 20-30 v/cm, and time of electrophoresis two to three hours.

Study with the ultracentrifuge shows that the preparations of gonadotropic hormones isolated from the urine of children by adsorption on kaolin with later frequent reprecipitations are relatively low molecular weight proteins which under the conditions of the experiment do not show heterogeneity from the presence of high molecular weight proteins. The use of adsorption on benzoic acid for isolation of urinary gonadotropins leads to getting heterogeneous hormonal preparations.

We express our thanks to Prof. Z. S. Gershenovich who has directed our study and to Prof. V. N. Orekhovich who made it possible to carry out the investigation with the untracentrifuge in the institution which he directs.

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† Original Russian pagination. See C. B. translation.

THE ENZYMATIC OXIDATION OF PHLOROGLUCINOL IN PLANTS

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The study of the transformations of phloroglucinol in plants is of considerable interest, since this polyphenol, besides occurring in the free state [1], is found in the molecules of a whole series of phenolic substances such as catechins, flavins, anthocyanins and other compounds [2] which play an important part in plant metabolism and which have great practical value. Unfortunately, there is little information on the transformations of phloroglucinol in plants.

Most of the work [3-11] is devoted to the biosynthesis of phloroglucinol and to study of the possibility of its transformation to other phenolic substances. There are separate observations on the enzymatic oxidation of phloroglucinol in plants. The work of James et al., [12] has shown a sufficiently energetic oxidation of phloroglucinol by an acetone preparation of polyphenol oxidase from belladonna. We have found oxidation of phloroglucinol by an acetone enzyme preparation from tea leaves [13]. It was established that at optimum pH values (5.3) for tea polyphenol oxidase [14] phloroglucinol oxidation did not occur, but at higher pH values oxidation became apparent. These results agree with the fact of energetic oxidation of phloroglucinol by acetone preparations from belladonna at pH 7.1, observed by James et al. In the work of Rubin and Ivanova [15] a suspension of cabbage leaves oxidized only phloroglucinol of all the polyphenols used by the authors.

EXPERIMENTAL AND DISCUSSION

The plant material was repeatedly treated with cold acetone to removal of the phenols and other acetone soluble substances; the residue served as the enzyme preparation. The intensity of oxidation of phloroglucinol by the enzyme was determined by absorption of oxygen in a Warburg apparatus. We were able to show that the ability for enzymatic oxidation of phloroglucinol was very widely distributed among the higher plants (Table 1).

This fact agrees well with the frequent finding in plants of phenolic substances in the composition of whose molecules phloroglucinol occurs. Lower plants, according to our data, also have a definite ability for enzymatic oxidation of phloroglucinol [13]. Aspergillus niger and Penicillium glaucum grown on extracts of tea leaves rich in catechins, that is, substances which contain phloroglucinol in their molecules, contain enzymes which oxidize phloroglucinol (Table 2).

The smaller amount of oxygen absorption in the case of phloroglucinol as compared to the oxidation of other phenols is evidently explained by the fact that pH 6 is not optimal for the enzymatic oxidation of phloroglucinol. This is shown in a study of the relation of enzymatic oxidation of phloroglucinol to pH. In carrying out these experiments, the composition of the samples was: 20 mg of acetone preparation from cabbage leaves, 3 mg phloroglucinol, phosphate buffer 0.015 M. The absorption of O_2 changed in the following way:

pH	O_2 absorption in microliters/hour
6	75
7	79
8	86
9	56

Oxidation of phloroglucinol by acetone preparations from cabbage leaves goes more intensively at pH 8.0. The higher optimum pH in the case of phloroglucinol as compared to the enzymatic oxidation of other polyphenols suggests that the enzyme which takes part in the oxidation of phloroglucinol may not be the same as the polyphenol oxidases which oxidize the other polyphenols. The probability of the idea of simultaneous existence in the acetone preparation of two polyphenol oxidases which differ from each other is partly confirmed by our experiments in which we compared the ability to oxidize phloroglucinol and pyrocatechol by the same acetone preparations, taken from different plants. As Table 3 shows, the ability to oxidize phloroglucinol and pyrocatechol with the acetone preparations from different

TABLE 1. Oxidation of Phloroglucinol by Acetone Preparations from Different Plants (reaction mixture: 50 mg of acetone preparation; 3 mg of phloroglucinol; phosphate buffer, pH 8.0)

Plant	O_2 absorption in microliters/hour
Tea leaves	48
Oak leaves	27
Aromatic tobacco leaves	45
Black currant leaves	29
White cabbage leaves	114
Potato tubers	113
Banana skin	41
Grape skins	11
Pear, fruit	98
Apple, fruit	28

TABLE 2. Oxidation of Polyphenol by Preparations of Polyphenol Oxidase of Aspergillus niger and Penicillium glaucum Grown on Extracts from Tea Leaves (reaction mixture: 10 mg preparation; polyphenols, 20 mg/mole; phosphate buffer, pH 6.0; O_2 absorption in microliters/hr)

Polyphenol	Aspergillus niger	Penicillium glaucum
Pyrogallol	60	55
Pyrocatechol	55	39
Phloroglucinol	35	25
Hydroquinone	44	38
Gallic acid	63	29
Tea tannin	68	54

TABLE 3. Oxidation of Phloroglucinol and Pyrocatechol by Acetone Preparations from Different Plants (reaction mixture: 50 mg of acetone preparation; 3 mg of polyphenol; phosphate buffer for phloroglucinol, pH 8, and for pyrocatechol, pH 6.0; O_2 absorption in microliters/hr)

Plant	Phloroglucinol	Pyrocatechol
Banana skin	41	205
Tea sprouts	48	79
Potato tubers	113	153
Cabbage leaves	114	0
Radish root	104	0

TABLE 4. Effect of Boiling and Diethyldithiocarbamate on Oxidation of Phloroglucinol by Acetone Preparations of Tea Leaves (reaction mixture: 50 mg of acetone preparation; 3 mg diethyldithiocarbamate, phosphate buffer, pH 6.0; 5 mg phloroglucinol; O_2 absorption in microliters)

Plan of experiment	1 hour	2 hours	3 hours	4 hours
Acetone preparation + phloroglucinol	20	50	84	114
The same + inhibitor	0	0	0	0
Boiled acetone preparation + phloroglucinol	0	0	0	0

plants is very different. The acetone preparations from cabbage and radish oxidize phloroglucinol quite energetically and do not oxidize pyrocatechol at all. The idea of the difference of enzymatic oxidation of phloroglucinol and pyrocatechol is also indicated by our finding of the fact that long keeping of the polyphenol oxidase from potato tubers causes full loss of ability to oxidize pyrocatechol and retention of the ability with respect to phloroglucinol. All these observations confirm the possibility of the existence of a special oxidase which oxidizes phloroglucinol.

In order to show that the oxidation of phloroglucinol in plants is not a purely chemical oxidation we inactivated the enzyme preparation by boiling or by addition of diethyldithiocarbamate as an inhibitor (Table 4).

The results show that addition of the inhibitor or heat inactivation of the enzyme produces full stoppage of phloroglucinol oxidation, indicating the great inertness of phloroglucinol to spontaneous oxidation by oxygen of the air and the existence in the plant of an enzyme which oxidizes phloroglucinol. This permits this polyphenol to take part in oxidation-reduction processes which occur in the plant cell.

SUMMARY

It has been shown that a whole series of higher plants, and also Aspergillus niger and Penicillium glaucum grown on extracts of tea leaves, have the ability for enzymatic oxidation of phloroglucinol. The optimum pH for the enzymatic oxidation of phloroglucinol is about 8.0. The oxidation of phloroglucinol in plants evidently occurs with an enzyme which differs from the polyphenol oxidase which oxidizes pyrocatechol, pyrogallol, and other polyphenols.

We express our thanks to Prof. M. A. Bokuchava for great aid in carrying out this work.

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ON THE SEPARATION OF METABOLIC PRODUCTS INTO FRACTIONS USING PAPER CHROMATOGRAPHY

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Paper chromatography is widely employed for the identification of various compounds in the study of biochemical reactions. This method, in conjunction with radioautography, has been frequently employed in the recent years by a number of workers in the field of photosynthesis. However, using this method difficulties were frequently encountered because of the poor separation of some compounds in undimentional chromatography, certain compounds frequently occupying identical positions in the developed chromatogram. For instance, with the solvent butanol acetic acid water (4:1:1), sucrose and glutamine run to the same position; in the solvent butanol/acetic acid/water (78:20:50) the pairs of compounds, glucose and glutamic acid, and alanine and fructose also run to the same positions. Even when two-dimensional chromatography is employed, some compounds still show unsatisfactory separation, making occassional rechromatography necessary.

The difficulty in identification naturally increases with the increasing number and variety of compounds in the mixture under investigation. On the other hand, diverse mixtures are the rule rather than the exception in the study of metabolic reactions. For instance, when leaves are exposed to light in the presence of $C^{14}O_2$ for even a short period (30 sec), more than 12 compounds with a high content of radioactivity can be identified [1]. The actual number of labelled compounds is considerably in excess of 12, but identification of some of them is rendered impossible because of the low degree of label.

Following a longer period of photosynthesis (1.5 min) the number of labelled compounds formed increases. Therefore, if identification is to be at all successful, it is necessary to carry out a preliminary separation of the mixture into classes of organic compounds formed in the various metabolic reactions: organic acids, amino acids, sugars, and organophosphates. Such a separation facilitates considerably the identification of the individual members of each group. Although within each fraction compounds still occassionally show similar behavior on some chromatograms, it is possible, using one or two solvents, to achieve a final separation of all the constituents of the fractions.

The present paper describes our attempts to develop a procedure for the chromatographic separation of the fractions mentioned.

EXPERIMENTAL

After checking a number of solvent systems, the following was found the most suitable for the purpose: propanol ammonia (sp. gr. 0.880) / 0.5% aqueous Trilon B (60:30:10 by vol.). The solvent has been recommended for the separation of phosphate esters [2].

We have found that, with this solvent system, a number of organophosphates could be separated from a mixture containing also sugars and amino acids. The separation is possible because of the slight solubility of the organophosphates in this solvents, so that they did not progress beyond 6 cm, while the front advanced 24.5 cm; under those conditions sugars and amino acids progressed considerably further than the organophosphates (Leningrad chromatographic paper No. 2 was used throughout, and chromatograms were run in the ascending direction). The distribution of various compounds on the chromatograms is shown in Fig. 1, while the R_f values were as follows:

Compound	R_f
Aspartic acid	0.34
Asparagine	0.37
Glutamic acid	0.41
Arginine	0.47
Histidine	0.54
Glycine	0.55
Lysine	0.55
Tyrosine	0.60
α-Alanine	0.60
Methionine	0.76
Tryptophane	0.85
Phenylalanine	0.88
Leucine	0.90
Raffinose	0.35
Maltose	0.43
Sucrose	0.50
Lactose	0.50
Glucose	0.52
Sedoheptulose	0.54
Fructose	0.55
Galactose	0.56
Ribose	0.58
Phosphopyruvate	0.11
Glucose-1-phosphate	0.13
Sodium glycerophosphate	0.16

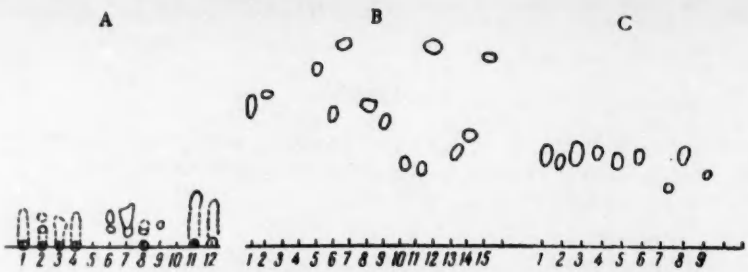

Fig. 1. The distribution of various compounds on chromatograms developed with propanol/ammonia (sp. gr. 0.880)/0.5% aqueous Trilon B (60 : 30 : 10) (by vol.). A: Organophosphates: 1) fructose-6-phosphate; 2) pyrophosphate; 3) orthophosphate; 4) ribose-5-phosphate; 5) fructose diphosphate; 6) glucose-1-phosphate; 7) glycerophosphate; 8) metaphosphate; 9) phosphopyruvate; 10) 2-phosphoglycerate; 11) 3-phosphoglycerate; 12) glucose-6-phosphate. B: Amino acids: 1) glycine; 2) tyrosine; 3) γ-aminobutyric acid; 4) β-alanine; 5) methionine; 6) histidine; 7) phenylalanine; 8) α-alanine; 9) lysine; 10) asparagine; 11) aspartic acid; 12) leucine; 13) glutamic acid; 14) arginine; 15) tryptophane. C: Sugars: 1) glucose; 2) sucrose; 3) fructose; 4) galactose; 5) lactose; 6) sedoheptulose; 7) raffinose; 8) ribose; 9) maltose.

It will be seen from the values quoted above that the R_f values of the organophosphates lay very close and that as a group they differed considerably from those of the other compounds, i. e., sugars and amino acids.

There is a scarcity of published information on the chromatographic separation of sugars and amino acids from mixtures. At the same time, the solution of this difficulty at present a laboratory technic of great importance. Existing methods for the separation of amino acids from sugars on ion-exchangers KU-1, KU-2 or other resins suffer from various disadvantages, the main being losses of amino acids.

The electrophoretic method for the separation of sugars from amino acids [3], apart from requiring special apparatus, calls for a number of precautions if errors are to be avoided.

Of the large number of solvents tested, only two systems were found suitable for the separation of mixtures of sugars and amino acids into a sugar and amino acid fraction. These solvents were as follows: 1) Pyridine/acetone/propanol/ammonia (sp. gr. 0.880)/0.5% aqueous Trilon B (60 : 10 : 18 : 9 : 3, by vol.), and, 2) pyridine/propanol/ammonia (sp. gr. 0.88)/0.5% aqueous Trilon B (60 : 24 : 12 : 4, by vol.). On the chromatographic paper employed by us (Leningrad No. 2, slow), both solvents ascended slowly, so that runs lasted 48 hours.

Tracings of chromatograms, showing the separation of amino acids from sugars, are presented in Fig. 2. It is clear that the chromatographic zones of the two groups of compounds are sufficiently widely apart, which permits accurate and reliable separation.

It is also known that in the process of metabolism there arise certain unstable compounds which are destroyed during the conventional analytical procedures and which require special mild isolation procedures. For instance, there have been recent references to some unstable compounds, formed during photosynthesis, which are destroyed during fixation with hot alcohol. These compounds were found to remain intact when photosynthesizing water-plants were fixed with cold solvents. The possibility of chromatography of such intermediates in the cold has not been investigated, and presented to us considerably interest.

We have therefore investigated the separation of a number of organic compounds using cooled solvents. This was achieved by the use of solvents incorporating liquid ammonia. The following solvent mixtures were used:

Solvent composition	Ratio by volume
Liquid ammonia – n-butanol	50 : 50
Liquid ammonia – butanol	100 : 50
As above	40 : 160
As above	160 : 40
Liquid ammonia – pyridine	40 : 160
As above	160 : 40
Liquid ammonia – methanol	40 : 160
As above	160 : 40

The solvents were prepared as follows: The non-ammoniacal component of the mixture (alcohol or pyridine) was placed first into the cylindrical jar which served as the chromatographic vessel, and was cooled with liquid nitrogen until crystals of the solid solvent appeared; the liquid ammonia was then added and ascending chromatograms were started immediately. The tightly closed jar was placed in a large Dewar flask containing liquid ammonia. Chromatography was thus carried out at the boiling temperature of ammonia −33°. Since the latent heat of boiling of ammonia is high (327.1 cal/g), evaporation was slight.

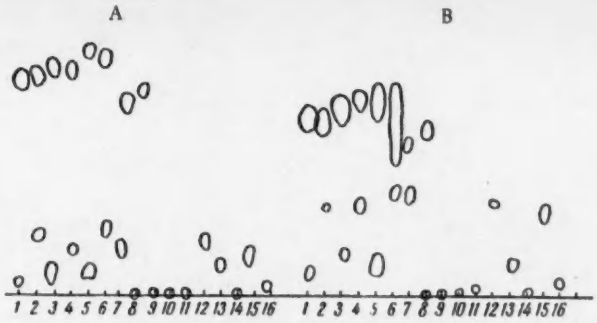

Fig. 2. The chromatographic separation of sugars from amino acids. A: 1) Glycine, glucose; 2) tyrosine, sucrose; 3) α-alanine, fructose; 4) methionine, galactose; 5) histidine, sedoheptulose; 6) phenylalanine, ribose; 7) leucine, raffinose; 8) aspartic acid, maltose; 9) asparagine; 10) glutamic acid; 11) arginine; 12) tryptophane; 13) serine; 14) cystine; 15) valine; 16) lysine; Solvent: pyridine/acetone/propanol/ammonia (sp. gr. 0.880)/0.5% Trilon B (60 : 10 : 18 : 9 : 3, by vol.). B: 1) Glycine glucose; 2) tyrosine, sucrose; 3) α-alanine, fructose; 4) methionine, galactose; 5) histidine, sedoheptulose; 6) phenylalanine, ribose; 7) leucine, raffinose; 8) aspartic acid, maltose; 9) asparagine; 10) glutamic acid; 11) arginine; 12) tryptophane; 13) serine; 14) cystine; 15) valine; 16) lysine. Solvent: pyridine–pyridine/propanol/ammonia (sp. gr. 0.880)/0.5% aqueous Trilon B (60: 24: 12: 4, by vol.).

When the above-mentioned solvents were employed, the phosphate compounds tested, 3-phosphoglyceric acid, fructose-1,6-diphosphate and glucose-1-phosphate, remained at the origin. The organic acids, citric, malic, succinic and fumaric, also remained on the origin. The amino acids tested, aspartic acid, alanine, glycine, and leucine, as well as the sugars, sucrose, glucose and fructose, are well soluble in ammonia and, in the solvent systems employed, they were well distributed throughout the chromatograms. It was particularly noted that the amino acids separated well, while the sugars, although they travelled sufficiently far, usually remained in a compact area. An example of the distribution of amino acids and sugars may be seen from the chromatogram tracing (Fig. 3 A).

The solvent containing pyridine and liquid ammonia (40 : 160 by vol.) deserved special mention; here the amino acids investigated were but sparsely soluble and travelled very little from the origin, while the more soluble sugars progressed considerably further (Fig. 3 B). This method therefore affords the separation of some amino acids from sugars by paper chromatography. Further development of mild chromatographic methods for the isolation of labile compounds using cooled solvents and, in particular, liquid ammonia, holds considerable promise.

Thus, once the most suitable solvents have been selected, the following separation methods can be suggested.

1. Separation into distinct fractions by a method using paper chromatography exclusively, without the application of ion-exchangers. This method would involve the following procedure; The initial step may be the conventional separation of the organic acids, either through extraction of the evaporated extract with sulfuric ether [7, 8], or by separation of the organic acids by paper chromatography using the solvent ether/formic (or acetic) acid/water (14 : 0.2 : 1.8) [9]. In the latter case the organic acids separate from the organophosphates, sugars and amino acids, all of which are insoluble in the solvent mixture and remain at the origin.

We have tried the following amino acids: aspartic acid, glutamic acid, glycine, serine, alanine, asparagine, glutamine, leucine. Sugars: glucose, sucrose, fructose, galactose, arabinose, mannose, lactose, raffinose, sedoheptulose. Organophosphates: Na glycerophosphate, ribose-5-phosphate, ATP, fructose-1,6-diphosphate and glucose-1-phosphate.

The portion of the chromatogram containing the organic acids may then be cut off the remaining compounds by sectioning approximately 0.5 cm from the line of origin. Caution must be exercised here, since some organic acids (oxalic, malonic, and gluconic acids) also remain on the origin, while certain other acids are sparsely soluble in this solvent and do not travel far from the origin [10, 11]. The organophosphates, amino acids, and sugars are eluted from the origin strip using the minimal volume of water. The eluate is then taken to dryness, the residue dissolved in a small amount of water and the solution applied to another chromatogram, which is irrigated with the solvent mixture propanol/am-

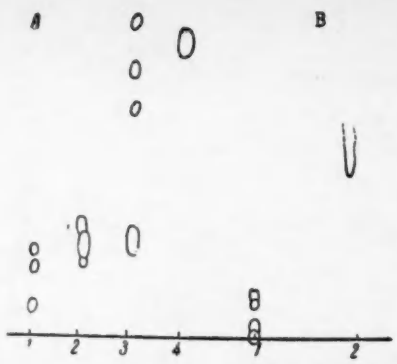

Fig. 3. Paper-chromatographic separation of some amino acids and sugars at very low temperatures. A: 1) Separated amino acids: aspartic acid, alanine, glycine and leucine; 2) unseparated mixture of sugars: sucrose, glucose and fructose. Solvent: liquid ammonia/ethanol (100:50 by vol.); 3) separated amino acids: aspartic acid, alanine, glycine, and leucine; 4) unseparated sugar mixture: sucrose, fructose, and glucose. Solvent: liquid ammonia/methanol (40:160 by vol.). B: 1) Amino acids: aspartic acid, alanine, glycine and leucine; 2) unseparated sugars: sucrose, glucose, and fructose. Solvent: pyridine/liquid ammonia (40:160 by vol.).

monia/0.5% aqueous Trilon B (60:30:10). The organophosphates remain close to the origin, travelling no further than 6 cm (as shown above). This portion of the chromatogram is cut off. The amino acids and the sugars are eluted from the remaining portion, the eluate is evaporated to dryness, dissolved in a small volume of water and reapplied to another chromatogram. The latter is irrigated with one of the solvents described above for the separation of amino acids from sugars. The chromatogram is again cut into two parts, the amino acids and sugars are eluted from the corresponding portions, and each fraction is then chromatographed separately for the separation of the individual components.

2. The organic acids are removed as described above. The remaining solution, containing organophosphates, sugars, and amino acids is passed through the cation-exchange resin KU-1 [12] or KU-2 [13]. The amino acids are retained and may be eluted with 6.0 N ammonia. The sugars and organophosphates remaining in the effluent may be separated from each other by paper chromatography, using the solvent propanol/ammonia/0.5% aqueous Trilon B (60:30:10 by vol.), as described above.

3. The chromatographic separation is carried out with the cooled solvents described above. In this case, the paper chromatography (in which lyophilized material is sometimes contraindicated [14]) is carried out with a specific solvent system which affords a separation of sugars and amino acids from the organic acids and organophosphates which remain on the origin. The origin strip is then cut off, both portions of the chromatogram are eluted and the eluates dried. Further separation of the mixed groups may be carried out by a variation of the above-described procedures, in combination with a variety of suitable solvent systems [15].

SUMMARY

A procedure for the separation of compounds of biological origin into fractions, using paper chromatography, has been developed. The solvent mixture, propanol/ammonia (sp. gr. 0.880)/0.5% aqueous Trilon B (60:30:10 by vol.) has been suggested for the separation of amino acids and sugars from organophosphates. Amino acids may be completely separated from sugars by paper chromatography, using the solvent pyridine/acetone/propanol/ammonia (sp. gr. 0.800)/0.5% aqueous Trilon B (60:10:18:9:3 by vol.), or pyridine/propanol/ammonia (sp. gr. 0.880)/0.5% aqueous Trilon B (60:24:12:4 by vol.). The separation of sugars from amino acids was particularly sharp when the former solvent mixture was employed. Refrigerant solvent mixtures, incorporating liquid ammonia, have also been suggested for the separation of organophosphates and organic acids from certain amino acids and sugars. A procedure for the separation of amino acids from sugars at low temperatures has also been described.

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†Original Russian pagination. See English translation.

UTILIZATION OF ACETATE FOR THE BIOSYNTHESIS OF CHACONINE AND SOLANINE IN POTATO SPROUTS

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The glycosidic alkaloids of the potato, α-solanine and α-chaconine, have the same aglycone, solanidine, but different carbohydrate residues.

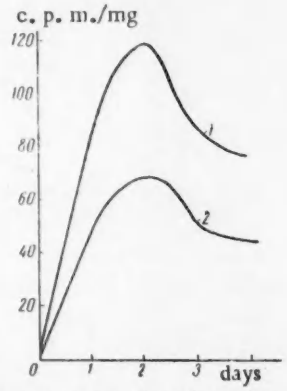
The structures of the sugars of α-chaconine and α-solanine have been elucidated by Kuhn, Low, and Trischman [1, 2]. The sugar of α-solanine is solatriose, 2-α-L-rhamnosido-3-β-D-glucosido-D-galactose. That of chaconine is chacotriose, 2-L-rhamnosido-4-α-L-rhamnosido-D-glucose.

We have shown in an earlier publication [3] that the C^{14} of $CH_3C^{14}OOK$ introduced into potato sprouts is utilized partly for the synthesis of the alkaloid glycosides. The object of the present research was to determine the distribution of the labeled carbon atom of $CH_3C^{14}OOK$ between α-solanine and α-chaconine.

METHODS

The experiments were performed during 1958 and 1959. Potato sprouts (Berlichingen variety) were placed in tap-water to which we added labeled potassium acetate (3 μmoles of $CH_3C^{14}OOK$, 200 μC per 100 ml of solution per 100 g of sprouts). The sprouts were removed at fixed intervals, washed, fixed, and ground to a powder.

The powder was extracted a number of times with boiling ethanol containing 1% of acetic acid, to extract the alkaloids. The extracts were evaporated to dryness under reduced pressure, the residue was dissolved in 1% hydrochloric acid, and the solution was filtered. A mixture of alkaloid glycosides was precipitated by addition of ammonia, collected by filtration, washed thoroughly with dilute ammonia, redissolved in 1% hydrochloric acid, and again precipitated with ammonia; this procedure was repeated a number of times. For the separation of the labeled alkaloid glycosides, and for the preparation of pure labeled α-chaconine and α-solanine, the mixture was fractionated on an Al_2O_3 column [4, 5], and the products were recrystallized from ethanol. The products so obtained were chromatographically pure [5].



Radioactivity of α-chaconine and α-solanine. 1) α-Chaconine; 2) α-solanine. Experimental conditions: 3 μmoles $CH_3C^{14}OOK$, 200 μC per 100 g of sprouts.

The purity of the products was checked by means of Alberti's color test [6], which showed that the radioactive preparations of α-chaconine and α-solanine were 100% and 99% pure, respectively.

We obtained 15.6 g of powder from 160 g of sprouts, and from it we prepared 0.24 g of alkaloid mixture. Chromatographic separation of the mixture gave 0.11 g of α-chaconine and 0.0847 g of α-solanine. The radioactivity of the products was measured; we did not apply any correction for the sensitivity of the counter, so that the results are of relative validity only.

The results, shown graphically in the figure, show that the specific activity of α-chaconine was higher than that of α-solanine. The activity of both the alkaloids rose progressively to a maximum at the end of two days, and then fell steeply. The activity of α-chaconine was at all times greater than that of α-solanine. The chaconine content of the sprouts usually exceeded that of solanine. The sprouts of Berlichingen potatoes were found to contain about 3% of chaconine and 2% of solanine. In our subsequent experiments we processed the sprouts after two days of exposure, since this ensured the maximum incorporation of radioactivity into the alkaloid glycosides.

The distribution of radioactivity between the aglycone and the trisaccharide residues was examined by hydrolyzing α-chaconine and α-solanine. The preparations were dissolved in N H_2SO_4 in 50 % ethanol, and the solutions were boiled under reflux for 7 hr. The solanidine thus liberated was precipitated with ammonia, collected on the filter, thoroughly washed with dilute ammonia, and examined by means of paper chromatography. The chromatograms of solanidine preparations thus isolated from either α-chaconine or α-solanidine gave only one spot, due to solanidine. The findings for the radioactivity

Specific Activity of Solanidine Prepared by the Hydrolysis of α-Chaconine and α-Solanine (experimental conditions: 3 mM $CH_3C^{14}OOK$, 200 μC per ml of solution per 100 g of sprouts)

Date of expt.	Alkaloid glycoside mixture	α-chaconine	α-solanine	Solanidine from chaconine	Solanidine from solanine
February, 1958	105	120	70	210	120
March, 1959	140	182	91	356	177

of the solanidine preparations are presented in the table.

It is evident from the table that the specific activity of solanidine prepared by hydrolysis of α-chaconine considerably exceeds that of solanidine from α-solanine. The higher radioactivity of α-chaconine than of α-solanine is due to the higher activity of its aglycone, solanidine. It may hence be concluded that the rate of formation and replacement of chaconine is greater than that of solanine.

Our results do not exclude the possibility of the existence of separate pathways of biosynthesis of solanine and chaconine. Addition of the sugars may take place at some stage earlier than that of formation of solanidine, and this stage may be different for solanine and for chaconine.

SUMMARY

It has been shown that [1-C^{14}] acetate is utilized by potato sprouts for the synthesis of alkaloid glycosides. The specific activity of α-chaconine was nearly twice that of α-solanine. The greater activity of α-chaconine is ascribable to the greater radioactivity of the aglycone solanidine of α-chaconine than of α-solanine.

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EXAMINATION OF THE LIPID CONTENT OF DIFFERENT PARTS OF THE LUMBAR ENLARGEMENT OF THE SPINAL CORD

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We have shown in our earlier publications [1-3] that the contents of dry matter, ash, total and nonprotein nitrogen, protein, and trace elements are different in different parts of the lumbar enlargement of the spinal cord. We concluded, from a study of the metabolic processes, that in the lumbar enlargement of the spinal cord the intensity of phosphorylative processes and of glucose and oxygen utilization, and the activity of enzymes of the anaerobic glycolytic sequence and of oxidation-reduction processes, are highest in the grey matter of the anterior columns. For other spinal tissues, these activities vary in the diminishing order: grey matter of the posterior columns, anterior root tissue, posterior root tissue, white matter. These activities were calculated on both a fresh and a dry weight basis.

It is known, however, that proteins, carbohydrates, and lipids are unevenly distributed in the tissues of the spinal cord, and that they have different metabolic activities. Lipids, which are the most metabolically inert components of spinal cord tissue, make up the bulk of the myelin. This is most plentiful in the roots and in the white matter. It is therefore necessary, for the evaluation of the metabolic rates of different parts of the spinal cord, to take into consideration their lipid content. We have accordingly determined the total lipid and cholesterol contents of the various regions of the lumber enlargement of the spinal cord for which we had previously recorded the above-cited metabolic data.

METHODS

Our experimental material was the lumbar enlargement of the spinal cord of cattle. It was collected from the Dnepropetrovsk Meat Combine within 7-12 min of slaughtering the animals by severing the carotid arteries. The procedure used for removal of the material, and the experimental conditions were as described earlier [1].

Total lipid content was determined by the method of Swanson and Artom [4], as modified by us and by Blokhina [5]. The analytical procedure was as follows: the dried grey matter of the anterior column, and of the posterior column, the dried white matter, and the dried anterior and posterior roots (separately), were extracted sequentially with lipid solvents. The material was first extracted with acetone for 12 hr at 0°. The residues were then extracted with 95% ethanol for eight hours, then with 1:4 ether-ethanol mixture for four hours, and finally with 1:1 acetone-chloroform mixture for four hours on a boiling water bath. The various lipid extracts were pooled (separately for each part of the spinal cord), filtered, and the solvents were distilled off. The residual lipids were dried at 50° for 30 hr. The residues of the extracts of the different parts of the spinal cord were then weighed.

Cholesterol was determined by the method of Engelhardt and Smirnov [6], with certain modification.

EXPERIMENTAL RESULTS AND DISCUSSION

The results found for the total lipid content of different parts of the lumbar enlargement of the spinal cord are presented in Table 1.

The data of Table 1 show that the lipid contents of the tissues analyzed may be arranged in the following descending order of magnitude: white matter, posterior roots, anterior roots, grey matter of the posterior column, grey matter of the anterior column.

The values found for the cholesterol content of the various regions of the lumbar enlargement of the spinal cord are shown in Table 2.

It is evident from these data that the tissues can be arranged in the same diminishing order of cholesterol content, both of fresh and of dry tissue, as of total lipid content. White matter is richest in cholesterol, followed by posterior roots, anterior roots, grey matter of the posterior columns, and grey matter of the anterior columns.

Thus of the various parts of the lumbar enlargement of the spinal cord, the lowest total lipid and cholesterol contents are found in the grey matter of the anterior columns, while the grey matter of the posterior columns has slightly higher contents of these constituents. As has been pointed out, these regions are distinguished by their high metabolic activity. The white matter of the spinal cord, which shows the lowest metabolic activity, is the richest in total lipid content. As is shown by the values given in Tables 1 and 2, the roots occupy an intermediate place with respect to their total lipid content, and the same applies to their metabolic activity.

It thus appears that the parts of the spinal cord which have the lowest lipid content are the most metabolically active. Recalculation on a basis of

TABLE 1. Total Lipid Content of Different Parts of the Lumbar Enlargement of the Spinal Cord (the values shown are the means of 10 experiments, as % of fresh weight; the figures in parentheses refer to % of dry weight)

White matter	Grey matter		Roots	
	posterior column	anterior column	posterior	anterior
		Mean		
23,87 (70,42)	13,88 (52,18)	13,47 (51,94)	20,13 (67,39)	18,94 (66,01)
		Minimum		
19,22 (62,87)	11,69 (52,10)	11,05 (45,92)	17,70 (63,45)	16,47 (60,97)
		Maximum		
27,54 (77,14)	15,52 (62,93)	16,22 (64,07)	22,24 (72,00)	21,95 (73,00)

TABLE 2. Cholesterol Content of Different Parts of the Lumbar Enlargement of the Spinal Cord (the values shown are the means of 12 experiments, as %, of fresh weight; the figures in parentheses refer to % of dry weight)

White matter	Roots		Grey matter	
	posterior	anterior	posterior column	anterior columns
		Mean		
3,43 (9,80)	2,79 (8,93)	2,46 (8,09)	1,67 (6,71)	1,59 (6,63)
		Minimum		
2,50 (8,69)	2,34 (8,00)	1,99 (6,66)	1,23 (5,55)	1,20 (5,88)
		Maximum		
4,66 (13,33)	3,38 (10,52)	2,86 (9,52)	2,82 (8,69)	2,14 (8,69)

lipid-free tissue (fresh and dry weight) of the intensities of glucose utilization, oxygen uptake, and phosphorylation, and of the summary activities of the enzymes concerned in anaerobic glycolysis and in oxidation-reduction processes, gives the same descending order of magnitude of metabolic activities, viz., grey matter of the anterior columns, grey matter of the posterior columns, anterior root tissue, posterior root tissue, and lowest of all, white mater of the spinal cord. This is as would be expected, since the regions with the highest metabolic activity have the lowest total lipid content.

SUMMARY

Determination of the total lipid content of different parts of the lumbar enlargement of the spinal cord applying sequential extraction of the tissues with different lipid solvents, showed that lipids are not uniformly distributed. The total lipid and cholesterol contents fall in the following descending order of magnitude: white matter, posterior roots, anterior roots, grey matter of the posterior columns, and grey matter of the anterior columns.

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EFFECT OF CAROTENE ON THE PHOTOCHEMICAL PROPERTIES OF CHLOROPHYLL

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It is known that light absorbed by the carotenoids of a number of photosynthesizing organisms may be utilized for photosynthesis; the energy of the light quanta absorbed by the carotenoids is most probably transmitted to chlorophyll a, which participates directly in photosynthetic processes. The findings of Sapozhnikov et al., [1, 2] point to the possibility that carotenoids may take part in the reactions of oxygen transport involved in the photosynthetic process.

Little is known, however, about the actual nature of the photochemical reaction between carotene and chlorophyll. Aronoff and Mackiney [3] showed that illumination of a solution of chlorophyll in acetone or benzene in the presence of oxygen leads to the irreversible oxidation of chlorophyll; addition of carotene to the solutions substantially retarded this process. Pepkowitz [4] found that exposure of acetone extracts of green leaves to sunlight led to the destruction of carotene content of carrot extracts was unaffected by illumination, but addition of chlorophyll to the extracts was followed by a fall in their carotene content. The same effect was found when pure carotene was added to solutions of chlorophyll in petroleum ether [4]. Lynch and French have shown that extraction of carotene from lyophilized chloroplast preparations causes a considerable fall in their photochemical activity [5]. Fujimori and Livingston found that in the presence of carotene and of oxidants the half life of the triplet state of the chlorophyll molecule in solutions is shortened, as was shown by measurements made with the aid of the flash technique [6].

We have already shown [7] that exposure of chlorophyll solutions to high-energy flash illumination may lead to photoreduction of chlorophyll by molecules of solvent or of other substances present in the solutions. This effect explains the action of oxidants, which react rapidly with the photoreduced forms of chlorophyll. Our experiments showed that carotene inhibited the photoreduction of chlorophyll, apparently by reacting reversibly with the photoreduced form of the pigment, as we have reported in a previous paper [8].

The object of the present research was to make a systematic study of the effect of carotene on the photochemical reactions of chlorophyll which have been examined in our laboratory. We first of all investigated the capability of carotene to undergo reversible transformations when exposed to light.

METHODS

We used β-carotene from the Krasnodar Vitamin Plant (recrystallized from benzene), and preparations of chlorophyll a + b and a, extracted from nettle leaves, and purified chromatographically. The experiments were performed with solutions of carotene in organic solvents, with colloidal solutions of carotene in water, prepared by the procedures described earlier [9], with suspensions of crystalline carotene in buffer solution, and with natural carotene-protein complex (carrot homogenate). All the solvents used were redistilled immediately before the experiments.

The experiments on the photosensitized oxidation of ascorbic acid were done in a Warburg micromanometer at 25°, using manometer vessels of a special design [10]. The vessels were illuminated from below by a 200 w incandescent lamp, through a filter which excluded the ultraviolet part of the spectrum (GG-1, for experiments with carotene), or through a red filter (RG-2) for experiments with chlorophyll.

All the experiments on the photoreduction and photooxidation of carotene and chlorophyll, as well as on the chlorophyll-sensitized photoreduction of methyl red, were performed in Thunberg tubes. The tubes were illuminated at the focus of the condenser of 300 or 500 w lamps, interposing a red filter in experiments with chlorophyll, and without a filter in experiments with carotene; illumination was effected in a specially designed ultrathermostatically controlled container, ensuring constancy of temperature during the operation. In most of the experiments the intensity of illumination amounted to 10^4 erg/cm^2/sec. The measurements were made on a SF-4 spectrophotometer, fitted with a well which permitted of the introduction of test tubes and of Thunberg tubes.

EXPERIMENTAL

Photochemical Properties of Carotene

Photoreduction

We examined the possibility of photoreduction of β-carotene by ascorbic acid in various media, taking into account the earlier observed effect of the basicity of the medium on the course of this type of reaction. The following media were used: ethanol, ethanol-H_2SO_4 mixtures, pyridine, piperidine, and glacial acetic acid. We added 10 mg of ascorbic acid to the solutions of

carotene in the various solvent media, evacuated the tubes, and exposed them to light for 3 min.

The carotene concentration used in these experiments was 0.8×10^{-5} M (the extinction coefficient, K, at the absorption maximum was about 0.7). We could detect no changes in the absorption spectrum of carotene after illumination at room temperature. Photoreduction of carotene was not found after illumination of its pyridine solution with ascorbic acid at -40°. It thus appears that β-carotene is not subject to photochemical reduction. The possibility of irreversible oxidation of β-carotene during illumination in the presence of air was previously known to exist.

Photosensitizing action. It has been shown in our laboratory that chlorophyll and its analogs powerfully photosensitize the oxidation of ascorbic acid [10]. The activity of β-carotene, in various physical states, in this type of reaction was examined. We examined the action of true and colloidal solutions of β-carotene, suspensions of crystalline carotene in buffer solution, and natural carotene-protein complex (carrot homogenate).

The carotene concentration used in experiments with colloidal solutions was 0.2×10^{-5} M, and 0.3×10^{-3} M in experiments with carotene in acetone solution. Oxidation of ascorbic acid was measured in a Warburg apparatus at 25°. We placed 5 ml of carotene solution, or 5 ml of buffer solution containing 5 mg of crystalline carotene, in the main compartment of the manometer vessel, and 1 ml of solution containing 10 mg of ascorbic acid in the side-tube.

After 10 min of equilibration, the ascrobic acid solution was tipped into the carotene solution, and the manometers were read in the dark for 15 min, after which the systems were exposed to light for 20-60 min. The manometers were read every 3 min.

The experiments with colloidal solutions, suspensions of crystals, and carrot homogenates were conducted at various pH, from 4 to 9. Spontaneous oxidation of ascorbic acid was not observed at acid and neutral pH, and carotene, whether as colloidal solution or as suspended crystals, did not show any photosensitizing effect. Oxidation of ascorbic acid took place at pH 9, at the same rate in the light or in the dark, and in the presence or absence of carotene. The natural protein-carotene complex (carrot homogenate) catalyzed the oxidation of ascorbic acid irrespective of the pH of the medium, but no photosensitizing effect could be observed. Oxidation of ascorbic acid was not observed in systems containing acetone solutions of carotene, whether in the light or in the dark.

We also investigated the ability of carotene to sensitize the photoreduction of methyl red by ascorbic acid. The oxidized form of this dye has an absorption maximum in acid solution at 520 mμ, whereas the reduced form shows no absorption at this wavelength.

The reaction was conducted in evacuated Thunberg tubes. Carotene was taken as an aqueous solloidal solution, and as a true solution in acetone. The optical densities were read at 30 sec intervals for the first 2 min, and then at 60 sec intervals for the next 10 min, with the tubes placed in the well of an SF-4 spectrophotometer.

Under our experimental conditions direct photoreduction of methyl red took place, but the reaction rate was unaffected by the presence of carotene. Carotene thus has no photosensitizing effect on the reactions of oxidation of ascorbic acid or of reduction of methyl red.

Effect of Carotene on the Photochemical Properties of Chlorophyll

Photooxidation of chlorophyll. It is known that chlorophyll solutions undergo irreversible oxidation when illuminated in the presence of oxygen. We investigated the effect of carotene on photooxidation of chlorophyll in acetone solution. Acetone solutions of β-carotene and chlorophyll a + b were prepared. The chlorophyll concentration of the systems was 0.6×10^{-5} M (K_{chl} at 660 mμ was equal to 0.525). The effect of carotene was examined at three concentrations: 0.15×10^{-4}, 0.15×10^{-5}, and 0.15×10^{-6} M. The reaction rate was derived from the rate of fall of K for chlorophyll at 660 mμ after illumination. Readings were taken at 5 min intervals (Fig. 1).

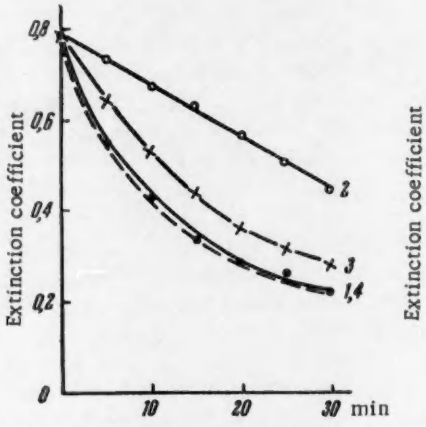

Fig. 1. Velocity of photochemical oxidation of chlorophyll in the presence of various amounts of carotene. $K_{chl} : K_{car}$ ratios: 1) 1:0; 2) 1:10; 3) 1:1; 4) 1:0.1.

Inspection of the curves of Fig. 1 shows that substantial retardation of the reaction of oxidation of chlorophyll resulted from the presence of carotene, in the proportion $K_{chl} : K_{car} = 1:10$; the effect of carotene diminished as its relative concentration fell, no effect being observed when the ratio was 10:1. It may be concluded that carotene protects chlorophyll from oxidation when acetone solutions of the latter are illuminated in the presence of air. At the same time, chlorophyll sensitizes oxidation of carotene, as was shown by the change in the absorption spectrum of carotene before and after illumin-

ation of the systems (Fig. 2). These results agree with the findings of the above-cited researches [3, 4].

Photoreduction of chlorophyll and pheophytin. We examined reduction of chlorophyll by ascorbic acid in the presence and absence of carotene.

Fig. 2. Differential spectrum during photooxidation of carotene (chlorophyll + carotene). 1) Before illumination; 2) after illumination.

For these experiments we took acetone and pyridine solutions of chlorophyll and carotene, in evacuated Thunberg tubes. The concentration of chlorophyll in the systems was 0.3×10^{-5} M (K at 660 mμ was equal to 0.53), and that of carotene was 0.2×10^{-5} M (K at 450 mμ was 0.53). The ascorbic acid content amounted to 10 mg per 5 ml of solution. The absorption spectra were measured over the range 700-400 mμ, before and after 30 min of illumination.

Appreciable reduction of chlorophyll (K fell by 0.265 after 30 min of illumination) took place in acetone solution, in the absence of carotene. In its presence there was practically no change in the value of K. It follows that carotene inhibits photoreduction of chlorophyll by ascorbic acid. No change could be seen in the absorption spectrum of carotene after 30 min of illumination, showing that carotene did not undergo sensitized reduction under these conditions.

In a second series of experiments we placed an ethereal solution of carotene into a Thunberg tube, evaporated off the ether, and added 4 ml of pyridine solution of chlorophyll a + b, chlorophyll a, or pheophytin, with 10 mg of ascorbic acid, as a reductant. The control systems were the same as these, but without carotene. The tubes were illuminated after evacuating air from them. The rate of photoreduction was derived from the fall in the value of K at 670 mμ during illumination.

The curves of Fig. 3 show that the rate of photoreduction of chlorophyll a falls in the presence of carotene. The inhibitory effect rises parallel with increase in the concentration of carotene. Carotene had a similar effect on the photoreduction of pheophytin a (Fig. 4), with a difference that relatively higher carotene concentrations were required. In order to ascertain the effect of lower

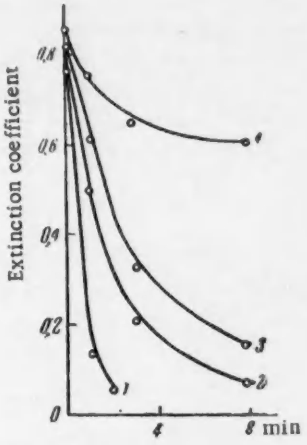

Fig. 3. Effect of varying the carotene concentration on the kinetics of photoreduction of chlorophyll a by ascorbic acid. Fall in the value of K at the absorption maximum of chlorophyll a (670 mμ) during the photoreduction reaction. 1) In the absence of carotene; 2) with 1.12×10^{-5} M carotene ($K_{470} = 1$); 3) 1.45×10^{-5} M carotene ($K_{470} = 1.3$); 4) 2.24×10^{-5} M carotene ($K_{470} = 2$).

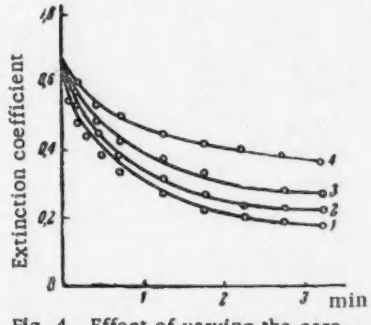

Fig. 4. Effect of varying the carotene concentration on the rate of photoreduction of pheophytin a by ascorbic acid. Fall in the value of K at the absorption maximum of pheophytin a (670 mμ) during the photoreduction process. 1) In the absence of carotene; 2) with 1.12×10^{-5} M carotene ($K_{470} = 1$); 3) with 2.24×10^{-5} M carotene ($K_{470} = 2$); 4) with 6.72×10^{-5} M carotene ($K_{470} = 6$).

concentrations of carotene on the photoreduction of pheophytin a, the experiment was repeated with a lower intensity of illumination (500 w, 40 v incandescent lamp), which slowed down the process, permitting of greater precision of measurement. Since carotene retards photoreduction, it was to be expected that it might react with the photoreduced forms of the pigments.

For this reason it could have been expected that addition of carotene solution to the photoreduced form of chlorophyll a would lead to accelerated regeneration of the initial pigment. It is, however, evident from Fig. 5

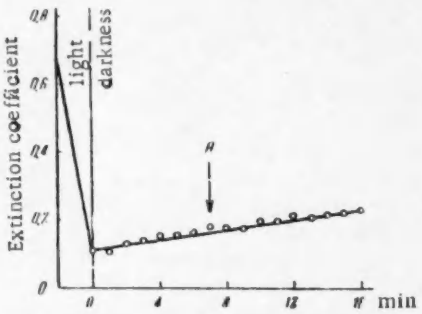

Fig. 5. Effect of carotene on the velocity of regeneration of the initial chlorophyll a from its photoreduced form. Increase in K at the absorption maximum of chlorophyll a in the dark, with progressive regeneration from the photoreduced form. A) Moment of addition of a pyridine solution of carotene from the stopper of the Thunberg tube.

that addition of carotene solution from the stopper of the Thunberg tube to the photoreduced form of chlorophyll a did not speed up regeneration of the initial form of the pigment. It follows that carotene does not react with the secondary photoreduced form of chlorophyll; however, the possibility of its reaction with the primary photoreduced form of chlorophyll is suggested by the experiments carried out in our laboratory by Evstigneev and Gavrilova [11]. Our experiments showed, however, that the primary reduced form of pheophytin a—protopheophytin—is readily formed in the presence of carotene. Evidently chlorophyll forms more active photoproducts, which are able to react reversibly with carotene.

Effect of Carotene on Oxidation of Ascorbic Acid, Photosensitized by Chlorophyll

We next compared the action of carotene in acetone or in colloidal solutions of different water contents (from 0 to 50%), in order to ascertain what effect the change in state of carotene from true to colloidal solution had on the reaction.

The pigments were dissolved in acetone. The carotene concentration in the systems was 0.28×10^{-5} M (K_{car} at 450 mμ was 1), and that of chlorophyll was 1.1×10^{-5} M (K_{chl} at 660 mμ was 1). A Warburg apparatus was used for the experiments. The main compartment of the vessels contained the solution of pigments in acetone, and the volume was made up to the required amount with acetone and water. A solution containing 10 mg of ascorbic acid was placed in the side-tube of the reaction vessel. The solutions were mixed, after equilibration, and oxygen uptake was measured for 15 min in the dark, and then for 21 min of illumination.

The results of these experiments are presented in Fig. 6. The curves show that in all the systems carotene had a greater or smaller inhibitory effect on the chlorophyll-activated photooxidation of ascorbic acid. The magnitude of this effect varies inversely with the water content of the systems, i. e., with transition from true to colloidal solutions.

Effect of Carotene on the Chlorophyll-Sensitized Photoreduction of Methyl Red

These experiments were preformed with true and colloidal solutions of the pigments.

The chlorophyll concentration taken in the experiments on true solutions was 0.3×10^{-5} M (K at 660 mμ = 0.28). The $K_{chl} : K_{car}$ ratio was varied from 0.1 to 10. The same amount of ascorbic acid was taken in all the experiments (10 mg in 0.5 ml water), as was that of methyl red. The systems all had a volume of 5 ml. The reactions were conducted in evacuated Thunberg tubes. Systems were illuminated at 30 sec intervals. Reduction of methyl red was measured from the fall in the extinction coefficient at 520 mμ, read directly in the Thunberg tubes.

The results of one of the experiments are represented in Fig. 7; the curves show the effect of carotene on the photoreduction of methyl red in acetone solution. Carotene caused substahtial inhibition of the reaction when the $K_{chl} : K_{car}$ ratio = 1 : 10 (curves 1 and 2) the effect was much smaller when the ratio was 1 : 1 (curve 3), and no effect could be seen when the ratio was 10 : 1 (curve 4). The existence of an induction period, suggested by the S-shaped curves, may be ascribed to the presence of oxygen in the media, and to its removal by sensitized reduction by ascorbic acid. A comparison of the absorption spectra of carotene before and after the experiments showed that carotene was not appreciably changed during the experiments. The procedure adopted in experiments with colloidal solutions was somewhat different.

The pigments were in colloidal solution in media consisting of 20% of acetone and 80% of water, and the systems contained 10 mg of ascorbic acid and equal amounts of methyl red. The systems, of a total volume of 5 ml, were in evacuated Thunberg tubes.

Air was pumped out of the tubes for 3 min. The concentration of chlorophyll was 0.6×10^{-5} M in all the experiments. The effect of carotene was examined at three concentrations, 0.15×10^{-4}, 0.15×10^{-5}, and 0.15×10^{-6} M. The systems were illuminated for 3, 5,

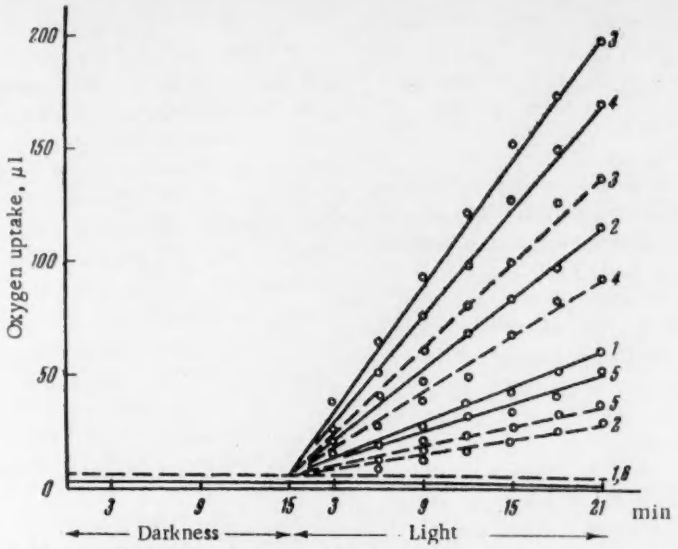

Fig. 6. Effect of carotene on oxidation of ascorbic acid, photosensitized by chlorophyll, in aqueous acetone systems. 1) Without water; 2) 10% water; 3) 30% water; 4) 40% water; 5) 45% water; 6) 50% water. Continuous lines – chlorophyll without carotene; broken lines – chlorophyll with carotene.

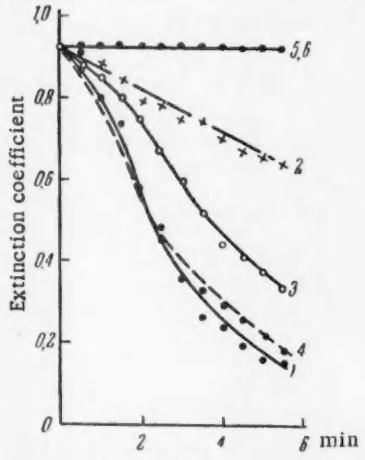

Fig. 7. Effect of different concentrations of carotene on the chlorophyll-sensitized photoreduction of methyl red. Fall in the value of K at the absorption maximum of methyl red (520 mµ) during its reduction. 1) In the absence of carotene; 2) with 0.8×10^{-5} M carotene ($K_{450} = 2.8$); 3) 0.8×10^{-6} M carotene ($K_{450} = 0.28$); 4) 0.8×10^{-7} M carotene ($K_{450} = 0.028$); 5) control experiment with chlorophyll, in the dark; 6) control experiment with chlorophyll and carotene, in the dark.

and 7 min. After exposrue to light, the colloidal systems were coagulated by addition of 0.1 ml of saturated aqueous $MgCl_2$, and were then filtered. The optical density of the filtrates was read at 520 mµ. Preliminary tests had shown that the initial value of K was not affected by coagulation of the colloidal pigments, i. e., that methyl red was not carried down together with the coagulated pigments from colloidal solutions.

These experiments showed that carotene has no effect on the chlorophyll-sensitized photoreduction of methyl red in colloidal systems, i. e., that reduction of methyl red proceeds at the same rate in the absence or the presence of carotene in various concentrations in the reaction mixture. Under the given conditions, we found that the value of K also fell in the unilluminated control systems during the experiment.

DISCUSSION

A study of the photochemical properties of carotene showed that it has no activating effect in the photoreduction and photosensitization reactions examined. We found, however, that carotene has a pronounced inhibitory effect on the following photoreactions of chlorophyll: photooxidation by atmospheric oxygen, photoreduction by ascorbic acid, and photosensitization of oxidation – reduction processes. This inhibitory effect is found only when the chlorophyll and the carotene are present in homogeneous solutions; colloidal carotene has no effect on the activity of colloidal chlorophyll.

The results of the studies on the mechanism of the photochemical reactions of chlorophyll conducted in our laboratory suggest that both physical and chemical mechanisms are concerned in the inhibitory effect of carotene.

"Physical" mechanisms. The reaction of carotene with chlorophyll in the excited singlet state should be associated with quenching of the fluorescence of chlorophyll by carotene. We could not, however, observe any such effect.

The possibility that carotene may react with chlorophyll in its long-lived metastable triplet state has been examined by Fujimori and Livingston [6]. As was pointed out above, however, the experiments of these authors dealt with elementary photoreduction, and the effect of carotene was on the length of the half-life of the primary photoreduced forms. The possibility thus remains that carotene may affect the intermediate formation of long-lived excited states of chlorophyll.

"Chemical" mechanisms. Here there exists the possibility of the reversible reaction of carotene with photoreduced forms of chlorophyll, such as, for example: $X + AH \rightleftharpoons \cdot X^- + \cdot AH^+$ (elementary photoreduction); $\cdot X^- + C \rightleftharpoons X + {}^{\cdot}C^-$ (transfer of an electron to carotene); $\cdot C^- + \cdot AH^+ \rightarrow C + AH$ (regeneration of carotene), where X is chlorophyll, AH is an electron donor, and C is carotene. The postulated acceptance of an electron must be reversible, since we could find no evidence of any stable structural change in carotene following its exposure to light in the presence of chlorophyll and ascorbic acid, in vacuo.

A possibility of this sort is indicated by our experiments, in which we demonstrated the inhibitory effect of carotene on the photoreduction of chlorophyll [8], and by the work of Evstigneev and Gavrilova [11], showing the inhibitory effect of carotene on the formation of the primary reduced form of chlorophyll. Further work is required for the elucidation of this question.

SUMMARY

Carotene does not activate reactions of photoreduction and photosensitization. Carotene inhibits all the photochemical reactions of chlorophyll which were investigated: photoreduction, photooxidation, and photosensitization of oxidation – reduction processes.

The inhibitory effect of carotene was manifested only when both carotene and chlorophyll were present in true solution; colloidal carotene had no effect on the photoreactions of colloidal chlorophyll.

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BIOSYNTHESIS OF VITAMIN B_{12} AND PORPHYRINS IN PROPIONIBACTERIUM CULTURES

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The hypothesis that the same basic biosynthetic steps are common to both vitamin B_{12} and porphyrins, based on the presence of certain structural similarities between the molecules of these substances [1, 2], may now be considered to have been established in principle. Incorporation into the vitamin B_{12} molecule of C-labeled acetate and glycine [3], δ-aminolevulinic acid [4, 5], and porphobilinogen [6], is evidence that these porphyrin precursors are also utilized for the biosynthesis of vitamin B_{12}.

The mere establishment of these facts can, however, be regarded as being no more than a starting point for the investigation of this problem, since the structural differences between the molecules of vitamin B_{12} and the porphyrins necessitate the postulation of different biosynthetic pathways. For our purposes, the greatest interest attaches to the recognition of the common biosynthetic steps, and to the identification of the stage at which the two biosynthetic pathways diverge, one of them then leading to the biosynthesis of vitamin B_{12}.

We chose Propionibacterium shermanii as the biological material for our research, for the following reasons: Firstly because this organism synthesizes large amounts of free porphyrins, in addition to vitamin B_{12}, which makes it of particular value for our purposes, and, secondly, because propionic acid bacteria may be used for the production of crystalline vitamin B_{12}, for which reason the study of vitamin production by this organism is of practical interest.

METHODS

The techniques employed for the preparation of inocula and for the assay of vitamin B_{12} were as described in an earlier paper [3]; porphyrins were determined by the method of Pawelkiewicz [7]. Qualitative analysis [8] of the bacterial porphyrin mixture showed that it consisted almost exclusively of coproporphyrin, with only trace amounts of other porphyrins (protoporphyrin).

The organisms were grown under the conditions described earlier [3], with the sole difference that the cultures were fortified after 48 and 72 hr of fermentation by addition of sterile glucose solutions (2 g/100 ml at 48 hr, and 1 g/100 ml at 72 hr), and that 5,6-dimethylbenzimidazole was not present in the initial culture medium, but was added in sterile aqueous solution after 72 hr of incubation. The total fermentation time was 96 hr. Under these conditions the yield of vitamin was raised by a factor of 2.5-3, amounting to 7-8.5 mg/liter. The yield of bacteria was measured by weighing the dried bacterial residues after extraction of vitamin. In addition to the ordinary peptone medium used in our laboratory [3] we used a corn medium, of the following composition: corn steep liquor 20 g (wet weight), glucose 20 g, $(NH_4)_2SO_4$ 2 g, $CoCl_2 \cdot 6H_2O$ 10 mg, in water, to 1 liter; the pH was adjusted to 6.5 by addition of 30% NaOH solution.

Free iron was determined colorimetrically, by the αα'-dipyridyl reaction [9], without preliminary ashing; the medium was first decolorized with activated charcoal.

Cobalt porphyrin was extracted with butanol from the medium, after saturation with $(NH_4)_2SO_4$, as for determination of prophyrins, the butanol was distilled off under reduced pressure, the residue was dissolved in water, the solution was heated with $KHSO_4$, in order to mineralize the cobalt, which was determined colorimetrically, from its reaction with nitroso-R salt [10].

RESULTS AND DISCUSSION

Effect of δ-Aminolevulinic Acid (ALA) on the Synthesis of Vitamin B_{12} and Porphyrins

We assumed, as a working hypothesis, that the initial stages of biosynthesis of porphyrins and of vitamin B_{12} are identical, and that a prophyrin precursor, such as is ALA, is equally a precursor of vitamin B_{12}. In order to verify this hypothesis we synthesized ALA by Shemin's method [11] (method A), and examined the effect of adding it to Propionibacterium cultures. We found that addition of 5-20 mg of ALA per 100 ml of peptone nutrient medium caused a three- to six-fold increase in the yield of porphyrins, without in any way affecting the yield of vitamin B_{12}; the organisms readily convert added ALA into porphyrins, but do not utilize it for the synthesis of vitamin.

In view of this finding, we attempted to modify the process in favor of vitamin synthesis, i. e., we attempted to force the organisms to metabolize added ALA by a different pathway to that commonly taken. With this object, we examined the effects of cobalt and iron on the systems; it is known that cobalt, which is a component of vitamin B_{12}, stimulates its production by the bacteria [1], and at the same time depresses production of porphyrin compounds, such as the ferro- and ferri-heme enzymes [12]. The depressive effect of Fe on the biosyn-

thesis of porphyrins is even more pronounced than is that of Co [13, 14]. Cobalt and iron were added to the nutrient medium at the same time as ALA.

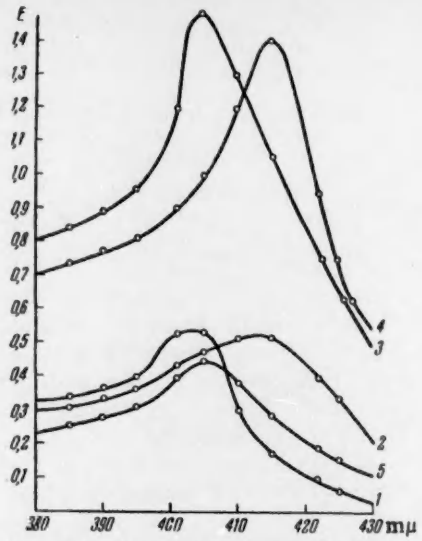
We found that in the absence of Co the same large amounts of porphyrins accumulate in the cultures, but that no vitamin b_{12} was produced. Addition of the least amount of cobalt (1 mg of $CoCl_2 \cdot 6H_2O$ per 100 ml) sufficed for normal production of the vitamin. Probably cobalt contributes in some way to the achievement of the final stages of formation of the cyclic structure of the vitamin; in the absence of cobalt free porphyrins only accumulate, possibly together with other, as yet unidentified intermediate products of a porphyrinic nature. The hypothesis that cobalt has such a complex-forming, coordinating role has already been advanced in the literature [15]. The yield of vitamin was not increased by raising the concentration of cobalt in the medium, which had, however, the effect of modifying the structure of the porphyrins produced; addition of 10 mg of $CoCl_2$ per 100 ml of medium was followed by substantial changes in the absorption maximum of the porphyrins, which showed a shift from 401-405 mμ to 415 mμ.

In all probability, under these conditions cobalt, with its strong tendency towards complex formation, entered into the structure of the porphyrin molecule, to yield a metalloporphyrin. The ease with which a reaction of this sort can proceed is evidenced by the findings of McConnel [16], who was able to prepare a number of Co-porphyrins by treating porphyrins with salts of cobalt. It is of interest that the absorption maximum of one of her Co-porphyrins was at 415 mμ, which coincides with out findings.

One of the characteristic properties of free porphyrins is that they can be extracted by ether from their acid aqueous solutions [8] (in our case, from culture media); this property was lost following incorporation of cobalt into the molecule. The presence of cobalt in the metalloporphyrins was confimred qualitatively by the nitroso-R-salt test. It may be concluded from all these data that large amounts of Co-porphyrins are in fact synthesized in cultures of Propionibacterium in media of high cobalt content.

As for the action of iron, we found that a concentration of 200 μg per 100 ml (as $FeSO_4 \cdot 7H_2O$) caused a substantial lowering of porphyrin production, which was totally suppressed by the presence of 1000 μg Fe per 100 ml, even in the presence of so effective a precursor of porphyrin synthesis as is ALA. Suppression of porphyrin synthesis was not, however, accompanied by the expected increase in the yield of vitamin B_{12}. The results of this part of our research are depicted graphically in the figure.

Thus, at the concentrations examined, addition of Co or Fe to the basic peptone medium did not cause any diversion of the biosynthetic reactions of the cells towards increased production of vitamin B_{12} from added ALA, although the presence of these metals had a substantial effect on porphyrin production.



Absorption spectra of the synthesized porphyrins (in N HCl), in different culture media. 1) Control (original peptone medium, without Co, Fe, or ALA). Per 100 ml of basic medium we added: 2) 1 mg $CoCl_2 \cdot 6H_2O$; 3) 20 mg of ALA; 4) 20 mg ALA + 10 mg $CoCl_2 \cdot 6H_2O$; 5) 20 mg ALA + 10 mg $CoCl_2 \cdot 6H_2O$ + 5mg $FeSO_4 \cdot 7H_2O$. Yield of vitamin, in mg per liter: 1 and 3 – 0; 2 – 6.8; 4 – 6.6; 5 – 6.7.

Significance of Iron for the Biosynthesis of Vitamin B_{12}

δ-Aminolevulinic acid was utilized differently when the basic component of the medium was corn steep liquor. Practically no porphyrins were produced by propionic acid bacteria grown in this medium, however much ALA was added (up to 40-50 mg%). Addition of such amounts of ALA caused, however, a 40-50% rise in the yield of vitamin B_{12}. It was found subsequently that the same effect was given by concentrations of 5-10 mg%, or even of only 1-2 mg%, of ALA.

The identification of the regulators of cell metabolism concerned in this change in direction of biosynthesis towards higher vitamin production was thought to be of interest. The total absence of porphyrins from corn steep liquor cultures, and the inhibitory action of iron salts on porphyrogenesis, suggested that the effect in question might be due to the high iron content of corn steep liquor. Determination of the iron contents of peptone and corn steep liquor media confirmed this supposition. We found that the corn steep liquor medium contained 102 mg of Fe per liter, whereas the peptone medium contained only 1.14 mg of Fe per liter, i. e., about a hundredth of the amount.

This explains the complete suppression of porphyrin synthesis in corn steep liquor cultures, and hence, possibly the utilization of added ALA for synthesis of additional amounts of vitamin. The impression received was that

Fe functioned here as a kind of regulator of the directions of biochemical processes proceeding in the cells, diverting them towards higher vitamin synthesis. Further evidense in favor of this view was afforded by experiments with corn steep liquor medium which had been freed of iron by boiling with chalk and potassium phosphate [7].

The absence of iron was confirmed by the $\alpha\alpha'$-dipyridyl test. The results of these experiments are presented in Table 1. It is evident from the data of this table that addition of ALA to the original culture medium considerably raised the vitamin yield (by 35 or 50%), whereas no porphyrins were formed under these quite favorable conditions. The results found after elimination of iron from the medium were very different.

The yield of bacteria from iron-free cultures was 35% higher than from the original medium; porphyrin production now took place, the yields rising from 7.5 mg per liter when ALA was not added, to 30 mg per liter in its presence. Addition of ALA did not, however, raise the yield of vitamin, which remained at the same level as in the control cultures, although this level was substantially (45%) higher than with the original corn steep liquor medium. Thus, the findings presented in Table 1 confirm the supposition that iron plays a regulatory role in porphyrin metabolism, since only in its presence is added ALA converted into additional vitamin B_{12}. Excess of iron (original corn steep liquor medium) has, however, an inhibitory effect on growth, leading to lower yields of bacteria and of vitamin. We therefore attempted to find the lowest concentration at which the regulatory function of iron was exerted, but at which vitamin B_{12} production was not depressed. With this object, we added ALA and a range of concentrations of iron salts (sulfate, citrate, chloride) to iron-free corn steep liquor and to ordinary peptone media. The results of one of these experiments are presented in Table 2.

It is evident from the data of Table 2 that, within the given range of concentrations, iron had no effect on the yield of bacteria. The effect of the iron salts added to the media on vitamin production was not the same as that of the iron present in the original corn steep liquor. Addition of 2 mg of iron lowered vitamin production in peptone medium by 15%, and the yield fell progressively with further increase in the concentration of iron. Ferric sulfate and ferric chloride had much the same effects, at somewhat lower relative concentrations.

On the other hand, addition of amounts of iron up to 8 mg/liter to iron-free corn steep liquor medium did not lower the yields of vitamin. The yield of vitamin fell slightly when the concentration of iron reached 10 mg/liter, but in this case the effect of adding ALA was not the same as for the original corn steep liquor medium, i. e., presence of high concentrations of iron did not now lead to additional vitamin B_{12} production from added ALA. We are obliged to conclude that the problem is of much greater complexity than we at first thought. It seems probable that the form in which iron is present in corn steep liquor is of importance. As for the metabolic shunt, we now consider it to involve at least two associated, but not causally interrelated, phenomena: Inhibition of porphyrogenesis in corn steep liquor medium is in fact due to its high iron content, but the transformations of ALA peculiar to this medium are due to some other factors present in corn steep liquor. We have absolutely no evidence as to the nature of these factors.

Irrespective of these matters, however, our findings suggest that while ALA is ideally adapted as structural material for the building up of the porphyrin molecule, it is utilized only with difficulty, under as yet not fully elucidated conditions, for the biosynthesis of vitamin B_{12}. We conclude that the biosynthetic pathways of porphyrins and of vitamin B_{12} diverge at some very early stage, such as that of production of ALA, which would be utilized for the synthesis of vitamin after conversion to some derivative, rather than in the form used in porphyrogenesis.

This conclusion, based on our experimental findings, is in agreement with Shemin's purely speculative assumption [4], based on a consideration of the structural peculiarities of the vitamin B_{12} molecule; in view of the exceptionally high number of methyl groups substituted into the molecule, he concluded that its precursor is more likely to be some methylated derivative of ALA, rather than ALA itself. On these grounds, we thought it would be of interest to study the methylation processes associated with vitamin B_{12} biosynthesis.

Significance of Methylation for the Biosynthesis of Vitamin B_{12} and Porphryns

It is now generally accepted that methylation is achieved in living organisms by means of enzyme systems linked with various active forms of folic acid and p-aminobenzoic acid (PABA) [17], insofar as the latter is a component of the folic acid molecule. One of the most widely applied procedures for the study of the action of folic acid and PABA is to suppress their activity by the use of various antagonists (usually sulfonamides), and then to examine the effectiveness of various metabolites in overcoming the inhibition.

Apart from folic acid and PABA, the metabolites commonly used for this purpose include purine and pyrimidine bases and amino acids, such as methionine, lysine, serine, leucine, and others [18, 20]. The overwhelming majority of such researches have been done on microorganisms, and the results have been assessed on the basis of changes in the growth and development of the organisms. A number of workers have shown that folic acid does not in every case abolish inhibition due to sulfonamides, and that it does not invariably replace PABA as a growth factor [21-23]. These findings suggest that the function of PABA within cells is not exclusively that of being a component part of folic acid, but that it may also be involved in other enzymatic reactions.

TABLE 1. Effect of Presence or Absence of Iron on the Yields of Vitamin and Porphyrins from Cultures in Corn Steep Liquor Medium

Composition of medium	Original medium			Iron-free medium		
	porphyrins			yield of bacteria		
	yield of bacteria, g/liter	B_{12}, mg/liter	porphyrins, mg/liter	yield of bacteria, g/liter	B_{12}, mg/liter	porphyrins, mg/liter
Basic	3.2	4.5	None	4.5	6.5	7.5
Basic + 5 mg ALA	3.2	6.0	"	4.5	6.5	15.0
Basic + 10 mg ALA	3.2	6.7	"	4.5	6.6	30.0

TABLE 2. Effect of Different Concentrations of Iron Citrate on the Synthesis of Vitamin and Porphyrins (1 mg of $FeC_6H_5O_7 \cdot 3H_2O$ contains ~ 200 μg Fe)

Composition of medium	Peptone medium			Iron-free corn steep liquor		
	yield of bacteria, g/liter			B_{12}, mg/liter		
	yield of bacteria g/liter	B_{12} mg/liter	porphyrins, mg/liter	yield of bacteria g/liter	B_{12} mg/liter	porphyrins, mg/liter
Basic(I)	4.3	7.8	9.5	4.0	6.5	7.5
I + 10 mg ALA per 100 ml of medium (II)	4.2	7.6	45.0	4.0	6.3	25.0
II + 0.2 mg Fe/100 ml of medium	4.3	7.5	40.0	3.9	6.5	20.0
II + 1.0 mg Fe/100 ml of medium	4.1	7.3	35.0	4.0	6.6	15.0
II + 2.0 mg Fe/100 ml of medium	4.2	6.6	30.0	3.8	6.4	–
II + 4.0 mg Fe/100 ml of medium	4.3	5.9	25.0	4.0	6.5	–
II + 8.0 mg Fe/100 ml of medium	4.2	4.7	9.0	4.1	6.3	–
II + 10.0 mg Fe/100 ml of medium*	4.3	4.0	3.5	3.9	5.8	1.5

* The Fe content of this variant corresponds approximately with that of the original corn steep liquor medium.

TABLE 3. Effect of Sulfathiazole on the Biosynthesis of Vitamin B_{12} and Porphyrins

Composition of medium	B_{12}, mg/liter	Porphyrins, mg/liter
Basic medium (I)	7.6	17.5
I + sulfathiazole, 1 mg/100 ml (II)	5.7	13.2
II + folic acid, 200 μg /100 ml	5.5	13.0
II + folinic acid, 200 μg/100 ml	5.6	13.1
II + PABA, 2 mg/100 ml	7.6	18.5
II + methionine, 2 mg/100 ml	7.7	13.0
II + threonine, 2 mg/100 ml	5.7	13.5
II + serine, 2 mg/100 ml	5.8	13.4
II + choline, 2 mg/100 ml	5.7	13.3
II + betaine, 2 mg/100 ml	5.6	13.4
II + sarcosine, 2 mg/100 ml	5.5	13.2

We used sulfathiazole as an antagonist to PABA, and aminopterin as a folic acid antagonist.* These antagonists were added to the original peptone medium, and we examined their effect on the production of porphyrins and of vitamin B_{12}. The results of experiments on inhibition with sulfathiazole are shown in Table 3.

At a concentration of 1 mg%, sulfathiazole causes a 20-25% inhibition of vitamin and porphyrin production, without affecting the yield of bacteria. At higher concentrations of sulfathiazole the rate of growth of the bacteria and their yield were lowered.

Addition of PABA totally abolished the inhibitory effect of sulfathiazole, restoring B_{12} and porphyrin formation to the normal levels. Folic and folinic acids were ineffective in this respect. We also examined the effect of adding the following possible methyl group donors: methionine, threonine, serine, choline, betaine, and sarcosine. Of these substances, only methionine was effective in abolishing the inhibitory action of sulfathiazole on vitamin B_{12} production. In this connection, we found that addition of methionine to the basic medium raised the yields of vitamin by 10-15%.

Methionine did not abolish the inhibitory effect of sulfathiazole on porphyrin production. A similar inhibitory effect of sulfonamides on porphyrin (chlorophyll) production has been reported by Gershanovich [24] for wheat seedlings.

The effect of aminopterin, a specific antagonist of folic acid, was next examined, with the object of acquiring additional evidence in support of our hypothesis that the inhibitory effect of sulfathiazole is due to interference with methylation of vitamin B_{12} precursors. The results are presented in Table 4.

The yield of vitamin B_{12} was lowered by 20-25% in the presence of 5 mg% of aminopterin, which had no effect on the yield of porphyrins or of bacterial mass (Table 4). As was to be expected, PABA did not abolish this inhibition, whereas folic, and in particular folinic acid, were effective in this respect; of the other methyl group donors only methionine was effective. These results provide further evidence of the importance of methylation processes for the normal synthesis of vitamin B_{12}.

We should, in connection with our results, mention that Shemin et al., [25] have shown that the C-labeled methyl group of methionine is incorporated into the vitamin B_{12} molecule, but not those of betaine or choline. These authors believe that it is this methyl group which contributes the six "extra" methyl groups of the vitamin B_{12} molecule.

Our results are in full conformity with these findings, and they show that one of the possible, and probable, differences between the biosynthesis of vitamin B_{12} and of porphyrins is the methylation process, which plays an essential part in the building up of the vitamin B_{12} molecule. The conclusion may hence be drawn that one of the functions of folic acid is to participate in the biosynthesis of vitamin B_{12}. There is as yet no indication of the stage of biosynthesis at which folic acid contributes to the building up of the vitamin B_{12} molecule – whether at that of methylation of the initial ALA, or of methylation of the completed porphyrin structure, modifying it to that of vitamin B_{12}.

A further indication of the difference between the processes of biosynthesis of porphyrins and of vitamin B_{12} is provided by the different sensitivities of these two processes to x-rays.

A 48-hr culture was exposed to a radiation dose of 30,000 r, after which it was inoculated into a number of flasks.† Raising the dose of radiation to 60,000 r led to total inhibition of growth in cultures of such inocula. The results obtained are presented in Table 5.

We found a considerable (40%) fall in production of vitamin B_{12} by cultures grown from irradiated inocula. The high sensitivity to radiation of the process of biosynthesis of vitamin B_{12} has been noted in the literature [26]. It is noteworthy that, in contradistinction to this effect, irradiation of the organisms had no effect on porphyrin production. Addition of methionine restored vitamin production to practically normal levels (88% of normal); folic acid was less effective. The cultures of irradiated organisms utilized added ALA for porphyrin production only, the yields of the latter being more than doubled; vitamin production was not stimulated by ALA.

This effect is also of interest in the following connection. The absence of any rise in the yield of vitamin B_{12} following addition of ALA to cultures in peptone or iron-free media might be a result of the existence within the cells of some specific physiological threshold; in other words, that there is some upper limit of vitamin concentration which cannot be exceeded within the cell. The results of the irradiation experiments show that such an explanation would be incorrect, since ALA was not utilized for the production of additional amounts of vitamin B_{12}, although the rate of biosynthesis of the vitamin was below normal. Similar results were obtained when cultures in ALA-containing media were not fortified with glucose during growth, under which conditions the yields of vitamins fell to 3-4 mg/liter.

To revert to the effect of radiation, it appears, in the first place, to cause inactivation of methylation processes, without affecting the sequence of reactions of condensation of ALA leading to the elaboration of the porphyrin ring structure.

DISCUSSION OF RESULTS

The literature dealing with the biosynthesis of porphyrins is both voluminous and diversified. The structure of practically all of the intermediate products has been

† We are indebted to N. A. Pomoshchikova, of the Institute of Microbiology, Academy of Sciences USSR (Moscow), for irradiating the cultures.

* N. A. Andreeva, of our Laboratory, kindly supplied us with the aminopterin.

TABLE 4. Effect of Aminopterin on the Biosynthesis of Vitamin B_{12} and Porphyrins

Composition of medium	B_{12}, mg/liter	Porphyrins, mg/liter
Basic medium (I)	8.3	16.0
I + aminopterin, 5 mg/liter (II)	6.3	16.0
II + folic acid, 200 μg/100 ml	7.0	15.5
II + folinic acid, 200 μg/100 ml	8.2	15.5
II + PABA, 2 mg/100 ml	6.3	16.5
II + methionine, 2 mg/100 ml	8.4	15.0
II + threonine, 2 mg/100 ml	6.5	15.5
II + serine, 2 mg/100 ml	6.0	16.5
II + choline, 2 mg/100 ml	6.0	16.0
II + betaine, 2 mg/100 ml	6.2	15.5
II + sarcosine, 2 mg/100 ml	6.4	16.5

TABLE 5. Effect of Irradiation on the Biosynthesis of Vitamin B_{12} and Porphyrins

Composition of medium	Yield of bacteria, g/liter	B_{12}, mg/liter	Porphyrins, mg/liter
Unirradiated inoculum (I)	4.25	7.1	15.2
Irradiated inoculum (II)	4.30	4.2	16.1
II + ALA, 10 mg/100 ml	4.20	4.0	35.3
II + methionine, 10 mg/100 ml	4.30	6.2	15.4
II + folic acid, 300 μg/100 ml	4.30	5.3	15.3

established for the biosynthetic pathway leading from glycine and succinic acid to the complex porphyrin ring structure [11, 27].

Numerous enzymes, catalyzing the various steps of this pathway have been isolated and studied [28], and many of its reactions have been accomplished in vitro. It is to be expected that it will not be long before the entire process will have been reproduced in vitro.

In contrast to this, the study of the biosynthesis of vitamin B_{12} has barely been begun. There are, in this field, more hypotheses and speculative assumptions than there are facts and experimental findings. The porphyrin-like structure of the vitamin molecule leads one to expect and to search for similarities between the biosynthesis of vitamin B_{12} and of porphyrins. At the same time, however, the numerous important structural peculiarities of the vitamin molecule suggest that its biosynthetic pathways should also differ from those of typical porphyrins. It is natural that efforts should be made to ascertain where the similarity between these two processes ends, and where the difference begins, i. e., at what stage do the two processes diverge. From their work on incorporation of C^{14}-labeled ALA or porphobilinogen into the vitamin B_{12} molecule, Shemin [5] and Schwartz [6] concluded that the pathways diverge at a later stage than that of formation of monopyrroles. Pawelkiewicz [2] had somewhat earlier arrived at a similar conclusion. We do not find these arguments to be altogether convincing, inasmuch as the utilization of ALA and porphobilinogen for the biosynthesis of vitamin B_{12} has as yet been demonstrated only qualitatively, whereas absolutely nothing is known about the quantitative aspects of the problem, viz., to what extent these substrates are utilized for this purpose. The publications of these authors do not give any information regarding the percentage incorporation of the labeled atoms into the vitamin molecule; for this reason, the bare fact that ALA and porphobilinogen are incorporated into vitamin B_{12} tells us nothing about the extent to which these substances are specific precursors of vitamin B_{12}. It is quite possible that they are incorporated only after conversion into some derivative. From our viewpoint, the experimental results reported in the present paper also support the latter possibility. The extraordinary ease with which added ALA is utilized for synthesis of additional amounts of porphyrins, and the extreme difficulty with which it is utilized for the biosynthesis of vitamin, and even then only under some special, as yet not fully recognized, conditions (corn steep liquor medium), allow us to conclude that ALA is a natural and specific precursor of porphyrins only, but not of vitamin B_{12}. It appears that, in order for ALA to become a direct precursor of vitamin B_{12}, either the ALA molecule must undergo some transformation, or some modification must take place in the sieries of transformations which lead eventually to the construction of the vitamin molecule.

Our findings regarding the significance of methylation for, and the effects of x-irradiation on, the biosynthesis of vitamin B_{12} and porphyrins provide additional evidence of the differences between these two processes, but they give no information as to the stage at which these processes diverge; methylation may be achieved during the initial, ALA stage, or during the terminal one of reconstruction of the intermediate phorphyrin-like structure, to give vitamin B_{12}.

It is obvious that the problem of the biosynthesis of vitamin B_{12} can be solved definitively only by the establishment of the identity of its specific precursor (that giving high percentage incorporation of labeled atoms), by the isolation of enzymes catalyzing the individual reactions of the process of biosynthesis of the vitamin molecule, and, finally, by reproducing the process in vitro.

SUMMARY

A study has been made of the biosynthesis of vitamin B_{12} and of porphyrins in cultures of Propionibacterium shermanii, with the object of establishing the differences and the resemblances existing between these two processes.

Addition of δ-aminolevulinic acid to the cultures was followed by very large increases in the production of porphyrins, without any rise in vitamin B_{12} production except in corn steep liquor media, in which porphyrin production was totally suppressed. This effect may be due to the high iron content (100 mg Fe/liter) of corn steep liquor. Following removal of the iron from corn steep liquor the stimulating effect of addition of ALA on porphyrin production became apparent, while stimulation of vitamin B_{12} production was no longer evident. The absence, however, of such effect of iron when it is added to peptone medium or to media which have been freed of iron points to the existence of some other, as yet unknown factors.

Methylation processes are of great importance for the biosynthesis of vitamin B_{12}, but not of porphyrins. The process of biosynthesis of vitamin B_{12} is much more sensitive to x-irradiation than is that of porphyrin synthesis.

It may be concluded from our findings that ALA is not as specific a precursor of vitamin B_{12} as it is or porphoryrins. It might be supposed that the biosynthetic pathways of these two products diverge at the ALA stage, and that a methylated derivative of ALA serves as the direct precursor of vitamin B_{12}.

In conclusion, we wish to express our sincere and profound gratitude to Professor V. N. Bukin for his constant interest in this research, and for the valuable advice which he gave us during its prosecution, and to Laboratory Technician V. Plechkina for her help in carrying out the experiments.

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ELECTROPHORETIC STUDIES IN SERUM PROTEINS ELICITING PASSIVE ANAPHYLAXIS

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In recent years, new facts have been gathered about the nature of antibodies concerned in anaphylaxis. While it was previously considered that anaphylactic antibodies were precipitins [1-4], hemolysins [5], or substances of a special kind [6], it has now been shown that several types of antibodies play a role in the pathogenesis of anaphylactic reactions. Thus, the Artyus-Sakharov phenomenon in rabbits, and man's allergic reactions to yolk proteins and some other antigens, may be imputed to the sensitizers of a type of precipitins [7]. It is remarkable that a large group of anaphylactic reactions are elicited by nonprecipitating or so-called 'incomplete' antibodies (Marrack [8]). In this group belong those antibodies transmitting passive anaphylaxis in guinea pigs, but also a whole series of allergic antibodies in man of the reagin type [9].

The experiments of Fedorov and Gurvich [10] appear to be one of the most conclusive demonstrations of the difference between the precipitins and sensitizers which transmit passive anaphylaxis from rabbits to guinea pigs. These authors showed that when rabbits were immunized with protein preparations, made nonanaphylactogenic by special treatment, a large number of precipitin antibodies appeared in the blood, although these immune sera did not elicit passive anaphylaxis in guinea pigs. However, whenever rabbits have been immunized with native proteins, the majority of authors have shown a close relationship between the quantity of sensitizers and of precipitin antibodies in the serum. The elucidation of the nature of this process presents not only theoretical but also practical interest.

The results reported here are of electrophoretic studies of the hyperimmune sera of rabbit blood, with a complete identification of the protein antibodies contained in different fractions, that transmit passive anaphylaxis to guinea pigs.

METHODS

Immunization of the animals. Rabbits were immunized with normal horse serum, or with different proteins fractions of it. The protein solutions were administered internally in increasing doses three times a week for a period of three weeks (in the first week up to 3 mg of purified protein, in the second to 6 mg, in the third to 9 mg). Reimmunization was carried out once within one to six months, by the internal administration of the corresponding antigen, 15-20 mg of protein being given. Within five to eight days, as a rule, sera were formed with a high content of precipitin antibodies and sensitizers, eliciting passive anaphylaxis in guinea pigs.

Fractionation of the serum. Separation of the serum proteins was made on a starch block (1 × 13 × 26 cm), in veronal-barbital sodium buffer (pH 8.6), over a period of 48 hr with a gradient density of 17.3 v/cm, and a field strength of 15-20 ma. A small amount of Evans' Blue (T-1824) was added to the serum to allow the movement of the proteins on the starch block to be observed visually during the process of electrophoresis. Parts of the starch block containing the proteins were separated into 1 cm transverse sections. The same buffer solution was used for eluting the proteins from the block as for the electrophoresis. The amount of protein in each sample was determined by Lowry's method [11], and a curve was constructed of the distribution of the proteins on the block. The homogeneity of the separated protein fractions was tested by means of paper electrophoresis.

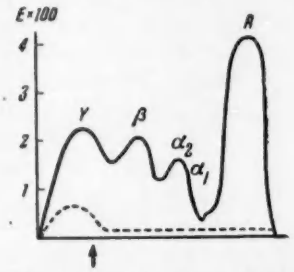
Identification of precipitins and sensitizers. The identification of the precipitin antibodies in the immune serum was carried out by Gurvich's electrophoretic-precipitation method [12]. The absolute amount of precipitins in the rabbit serum was found by the immunochemical method of Peters and Anfinsen [13], with this difference, that the amount of immune serum in the different protein fractions was found by the induction of passive anaphylaxis. A known amount of the different protein fractions (2-10 mg) was introduced into the stomach of a guinea pig weighing from 200-400 g. Within the next 24 hr, a determined dose of the corresponding antigen was introduced intravenously. The amount of sensitizers in each fraction was calculated by the minimal dose of protein, eliciting that degree of passive anaphylaxis, which, upon reinjection of the antigen, resulted in severe anaphylactic shock. Thus, it was possible to establish the absolute amount of precipitins and sensitizers in the proteins.

Preparation of precipitin solutions. The precipitation reaction was set up with different volumes of immune serum and the optimum concentration of antigen. It was carried out with electrophoretically homogeneous fractions of γ-globulins in order to isolate the precipitins. The precipitins, separated from the rest of the serum, and from the solutions of γ-globulins, were used to induce passive anaphylaxis. The precipitate formed after

The Detection of Passively Sensitizing Antibodies in Different Proteins Fractions of Hyperimmune Rabbit Serum

Days after reimmunization	Conc. of precipitins in rabbit blood serum, mg/ml	Proteins (mg) for sensitization (passive)	Fractions of immune serum proteins used for transmitting passive anaphylaxis				
			albumin	globulins			
				α_2	β	γ	γ-precipitins
0	0,32	10	—	++	+	+++	
				++	+	++	
6	9,00	4	—	++++	++	++++	+++
				++++	++	++++	++
8	7,70	3	—	++++	+	+++	+
6	5,04	3	—	++++	++	++++	++
					+		
11	3,60	3	—	+++	—	++++	++
				++	—	++++	+
6	4,32	4	—	++++	++	++++	++++
				+++	++	++++	++++
6	4,32	3	—				++
6	4,32	2	—				—

Designations: ++++ Severe anaphylactic shock resulting in death within 2-5 min; +++ pronounced shock with asthma, loss of ability to stand on the feet with subsequent asphyxiation and death within several hours; ++ clear anaphylactic reaction with the appearance of bronchial spasms; + anaphylactic reaction in the form of scratching of the snout, sneezing, bristling of hair, loss of activity; — without the apperance of characteristics of anaphylactic shock.



Electrophoretic curve of rabbit hyperimmune serum, and the distribution of the precipitate after special treatment of the electrophorogram. Dotted line — the position of the precipitate, with an arrow marking the origin of the serum. γ-, β-, α_2- α_1-globulins; A-albumen.

incubation in a thermostat at 38° for 25 min was carefully centrifuged and washed three times in physiological saline. The precipitate treated in this way was then dissolved in acid buffer solution (pH 2.2-2.4). Within 15-20 min, the precipitin solution was administered to a guinea pig. The reinjection of the antigen was carried out as described above.

RESULTS

In the figure is shown the curve of the electrophorogram of the immune serum before and after special treatment by the electrophoretic-precipitation method. As can be seen, the precipitin antibodies in rabbit serum migrate along with the γ-globulins. This also confirms that only solutions of γ-globulins form a specific precipitate with the corresponding antigen, while solutions of other protein fractions of immune serum do not posses this property.

The connection between the formation of precipitin and anaphylactic antibodies is clearly shown at the time of reimmunization. Thus, while by the thirtieth day after the first immunization the yield of precipitins reaches from 0.1-0.3 mg/ml, and it requires about 40 mg of globulins to cause severe shock by passive sensitizers, six to eight days after reimmunization, the content of precipitins has risen on the average to 6 mg/ml, and 3 mg of α_2 or γ-globulins are sufficient for passive sensitization.

Thus, at the same time as an increase in content of precipitins occurs in the reimmunization period, there is a parallel growth in the ability of the serum to elicit passive anaphylaxis. However, it does not follow from this that the ability of the immune serum to transmit passive anaphylaxis is due entirely to precipitin antibodies. Electrophoretic analysis shows that the migration of precipitins differs from that of some sensitizers. It follows from the table that it is possible to bring about passive anaphylaxsis by introducing solutions of α_2- and β-globulins. It is not possible to detect precipitin antibodies among the various fractions shown. Therefore, the sensitizers, migrating along with the α_2- and β-globulins can be referred to as "incomplete" antibodies. Solutions of γ-globulins may also transmit passive anaphylaxsis. It may be considered that precipitin antibodies have just the same ability to transmit passive anaphylaxsis as carefully washed precipitates dissolved in acid buffer solutions. However, the following important fact should

be noted. Solutions of γ-globulins, containing 3-4 mg of pure protein, or 1.2-1.6 mg of specific proteins (precipitins), elicit about the same effect as 3-4 mg of antibodies, which have been dissolved after precipitation. From this, two hypotheses can be made: Either the antibodies are greatly altered by precipitation, their anaphylactic activity being reduced, or "incomplete" antibodies with their corresponding sensitizers migrate along with the γ-globulins. There are indications that precipitin antibodies, separated after precipitation, to a great extent lose their precipitating characteristics [14]. It is possible that their ability to transmit passive anaphylaxis is also lost.

SUMMARY

In the serum of reimmunized rabbits, specific proteins (sensitizers) were found with the globulins, having the ability to elicit passive anaphylaxis in guinea pigs.

Sensitizers, migrating with α_2- and β-globulins can be related to "incomplete" antibodies. The sensitizers which migrate with the γ-globulins possess precipitating characteristics, but the possibility that there are also "incomplete" antibodies present is not excluded.

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AMIDE AND CARBOXYL GROUPS OF CEREBRAL PROTEINS DURING INTOXICATION

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The mechanism of cerebral protein participation in performance of a specific organ function depends on the ability of the protein molecules to react rapidly and reversibly with complexes formed during the metabolic process. The reactibility of protein molecules, in the first place, is determined by the presence and placement in them of active functional groups: Sulfhydryl, carboxyl, amide, amine, guanidine and others.

It has been ascertained that the biological activity of many proteins is determined by "active centers" which occupy but a small part of the protein molecule. The most intensly studied is the role of the sulfhydryl groups in the biological activity of different protein-enzymes. It can be assumed that other functional groupings, carboxyl groups for example, also perform a specific role in reactions of proteinaceous substances with various substrates.

Our prior experiments [1] have shown that under the influence of increased oxygen pressure, already during the first few minutes, there are released in the animal's brain large quantities of ammonia. At the same time the animal's behavior does not reveal signs of disturbance. The subsequently developing convulsions are accompanied by a ten fold or more increase in the brain ammonia. This coincides with the observations of other authors [2-5] concerning the connection between the functional state of the neural system and the release of ammonia, The physiological state of ammonia, freed during excitation, has not been fully explained and the majority of the authors tend to regard it only as a toxic substance, the immobilization of which in the organism has been accomplished by evolution of complex systems of chemical reactions.

Analyzing the possible biological importance of ammonia one can presuppose that one of the more primitive and important reactions of ammonia is its reactivity with proteinaceous substances. As a result of such reactivity there can arise either salt or amide linkages of the ammonia ion. In many biologically active proteins (vasopressin, oxytocin and other) the amide linkage is frequently encountered.

In order to clarify the relation between the functional state of the brain and the dynamics of amide and carboxyl groups of proteins we have studied the content of these groups in brain proteins of animals placed in the atmosphere of increased oxygen pressure. We have chosen oxygen as the natural factor of the medium, which, when its partial pressure in the surrounding atmosphere is elevated calls forth in the animal (depending on its pressure and time of action) states from deep sedation to violent convulsions.

EXPERIMENTAL

The experiments were conducted on white rats weighing 150-200 g. The animals were placed in a barometric chamber where pressures of pure oxygen of 6 or 3.5 atm were maintained. Alkali were used to trap the carbon dioxide. Under the influence of oxygen with pressure of 6 atm, rats begin to show excitation after 5-10 min, this becomes progressively intensified and after the 15-20th minute convulsions begin to appear. When the experiment is continued, the intervals between the bouts become shortened, the bouts become heavier and the animal perishes. The preconvulsion period of animals placed in 3.5 atm of oxygen pressure is 90 min or longer.

We have studied the amide and carboxyl groups of cerebral protein during periods of slight excitation, strong excitation and strong convulsions. This periodicity is of course somewhat artificial. The dominant state of the animal was taken as the basis. Control rats were kept under normal atmospheric pressure. In additon, for comparative purposes, we studied cerebral proteins from rats under short term ether narcosis (5-10 min). In rats ether narcosis ensues without prior excitation. When the required state was reached the experimental and control animals were decapitated simultaneously, the brains were rapidly extripated, the protein preparations were prepared and the "total" and water-soluble proteins were studied.

In order to obtain the sum of proteins, the brain was ground with 5 ml of 10% TCA solution, the precipitate was separated by centrifugation and freed from water and lipids by sequential washing with acetone, methanol-chloroform mixture (3 : 1), alcohol, mixture of alcohol and ether (2 : 1), and finally with ether. The preparation was air-dried and ground into a fine powder in an agate mortar. In order to obtain the water-soluble cerebral proteins acetone powders were prepared at –20°. After drying in a vacuum dessicator over P_2O_5 the powder was processed through a silk sieve. Powder weighing 100 mg was homogenized for 10 min with 4 ml of doubly distilled

water at cold temperature and left overnight in the refrigerator. The precipitate was centrifuged down and thrown away. The water-soluble proteins remained in the solution.

Determination of amide groups. The cleaving of all of the amide groups (AG) from cerebral proteins takes place after one to three hour boiling in diluted (2-5N) acid. However, in the make-up of the protein molecule there is evidently found a part of AG which is cleaved already after hydrolysis in 2N for 10 min. These AG can be called "labile", in deference to the "firmly-bound", which are cleaved only after further hydrolysis.

The determination of the labile and firm-bound AG was conducted in the total and water-soluble cerebral proteins of all the experimental animals. Both fractions were determined in the same protein sample. The sample of total proteins was homogenized in 2N H_2SO_4. After hydrolyzing for 10 min the vial was cooled, centrifuged and an aliquot part was hydrolyzed for three hours, and after that the proteins were precipitated with 0.5 ml of 20% TCA; the sample was centrifuged and ammonia was determined in the supernatant. Ammonia was determined by the microdiffusion method of Seligson [6]. The quantity of AG was calculated in mg NH_3-N/mg N protein in the sample.

Determination of carboxyl groups. Carboxyl groups were determined by esterification with methyl alcohol in the presence of thionyl chloride. This method avoids esterification of the hydroxyl groups and cleavage of the peptide bonds. This makes the method fairly specific for free carboxyl groups [7].

We used C^{14}-methyl alcohol and the number of free carboxyl groups was determined from its incorporation into the cerebral proteins. The sample of the total protein was homogenized with 40 volumes of absolute methyl alcohol transferred into a flask to which was added 0.25 μC of C^{14}-methyl alcohol. The flask was hermetically sealed with a rubber stopper through which was placed a calcium-chloride tube and a dropping funnel. The flask was placed in a cooling mixture (ice, salt, water). Stirring was accomplished with a magnetic stirrer. During 30 min one fourtieth of the volume of thionyl chloride was added dropwise. Methylation was continued for 3 hr with constant stirring. The protein was precipitated with 20% TCA, separated by centrifugation, and washed free of the radioactive waste. The precipitate was dried at room temperature, it was spread out and the radioactivity was determined from a thick layer by using a block counter. Radioactivity was expressed in terms of counts/min per 100 mg of protein.

EXPERIMENTAL RESULTS

The dynamics of the number of amide and carboxyl groups of total proteins. Animals under experiment were subjected to the oxygen pressure of 6 atm. The experiments were conducted on ten experimental and ten control animals. The experiment was terminated when the animals showed pronounced excitation; the mean time of the animals' sojourn in the chamber was 25 min.

The same sample of total cerebral proteins was used for the determination of free carboxyl and amide groups. In the control animals the sum of AG in total cerebral proteins was on the average of 73 μg NH_3-N/mg N protein (range 70.0-79.1 μg/mg). The incorporation of labeled methyl alcohol into the same proteins was at the average of 80.0 counts/min/100 g of protein. In the experimental animals the sum of the amide groups in the total proteins was on the average of 84.7 μg NH_3-N/mg N protein (range 78.2-91.1 μg/mg). The incorporation of labeled methyl alcohol into these proteins was on the average of 54 counts/min/100 mg of protein.

Thus, in animals that were subjected to oxygen pressure of 6 atm and which were in the state of strong excitation we have observed a slight increase (on the average 15%) in the number of AG in the total cerebral proteins with a concurrent decrease in the number of free carboxyl groups (on the average 34.4%).

AG of total and water-soluble cerebral proteins. Numerous investigations have shown that the brain contains proteins with various properties. These can be separated by salt and solvent fractionation or by electrophoresis.

The participation of varicus proteins in physiological processes is unequal. Starting from this, we have studied the change in the number of AG in water-soluble proteins as compared with total cerebral proteins of rats in different states, created by the action of increased oxygen pressure.

The sum of AG in cerebral protein. The changes in the number in cerebral proteins of rats in different stages of oxygen poisoning are shown in Fig. 1.

In the total cerebral proteins of the control animals the quantity of AG is on the average 73.6 μg NH_3-N/mg N protein. In the total cerebral proteins of rats subjected to increased oxygen pressure, during all of the periods of oxygen poisoning, the same average increase of 15.1% in the number of AG is observed.

A somewhat different picture is observed in the water-soluble proteins. In the control the sum of AG was 85.6 μg NH_3-N/mg N protein. Excitation of the animals in the beginning of oxygen action (7-8 min) leads to the average lowering in the number of AG of 31% as compared with the control. Further increase in excitation especially the appearance and development of convulsions leads to an increase in the number of AG. From Fig. 1 it is evident that different degrees of excitation are characterized by a large dispersal of points (from 77.1 to 131 μg). During the convulsion period the AG of water-soluble proteins comprise on the average 130.8 μg, i. e., an average increase of 64.3%.

From our previous work [1] it is known that the slightest excitation brought forth by increased oxygen pressure results in the increase of free brain ammonia. Development of oxygen poisoning, with concurrent convulsions,

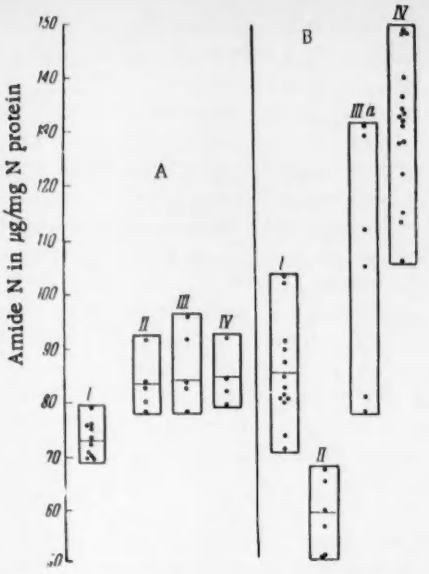

Fig. 1. Changes in the total number of AG in brain proteins of rats under oxygen intoxication. Oxygen pressure is 6 atm. A-Total proteins; B – water-soluble proteins. I – Control. II – moderate excitation; III – beginning of convulsions; IIIa-pronounced excitation; IV-strong convulsions.

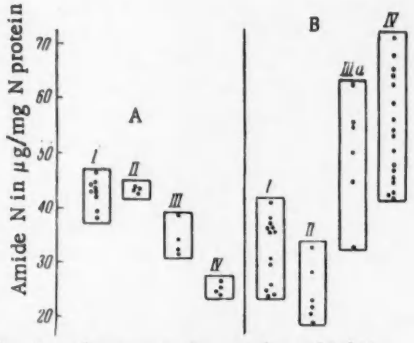

Fig. 2. Changes in the number of labile AG in cerebral proteins of rats under oxygen intoxication. For legend see Fig. 1.

leads to further increase in the amount of ammonia. It can be supposed, that during the first stages of oxygen poisoning the water-soluble cerebral proteins are the main source of ammonia. The subsequent great increase in the amount of ammonia depends on destruction of other nitrogenous compounds-glutamine, adenylic compounds, amino sugars et. With this surplus of ammonia the reaction is shifted toward its tying up by the free protein carboxyl groups. The possibility is not excluded that at first there is adsorption of the ammonia and then its conversion into AG.

Labile AG in cerebral proteins. Labile AG in total cerebral proteins of control animals (Fig. 2) comprised on the average 42.5 μg NH_3 N/mg N protein. During the period of excitation, produced by oxygen intoxication, the number of a labile AG remained almost unchanged and was on the average 43.8 μg. However, with the beginning of convulsions their number was decreased. The deeper and more prolonged were the convulsions, the smaller becomes the number of these groups.

The total number of AG in the total cerebral proteins during the same period of oxygen poisoning during the same period of oxygen poisoning remained almost unchanged (Fig. 1). In connection with this, the relation (expressed in percent) between labile AG in the protein and total AG of the same protein must change:

Control	Excitation	Convulsions	Strong convulsions
57.6	52.3	39.7.	29.5

In the water-soluble rat cerebral proteins under the action of oxygen pressure of 6 atm the dynamics of labile AG content reproduce the dynamics of total AG of total proteins. In this way, the relation of labile AG of the water-soluble rat cerebral proteins to the total AG content in the same protein, during the period of development of oxygen poisoning, remains unchanged. As expressed in percent this relationship was:

Control	Moderate excitation	Strong excitation	Convulsions
37.4	40.8	45.0	40.5

Firmly-bound AG in cerebral proteins. In Fig. 3 are presented the dynamics of the numbers of the firmly-bound AG of total and water-soluble rat cerebral proteins when the animals were subjected to oxygen pressure of 6 atm.

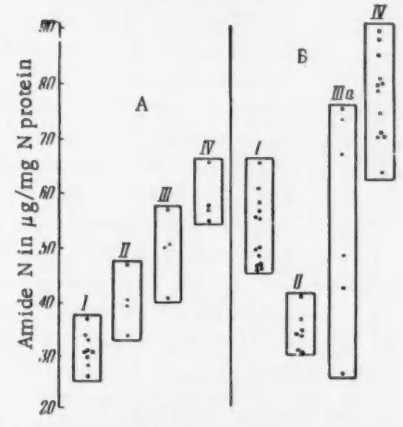

Fig. 3. Changes in the number of firmly-bound AG in cerebral proteins of rats under oxygen intoxication. For legend see Fig. 1.

The development of oxygen poisoning resulted in the increase in the formly-bound protein AG. The relation of the firmly-bound AG to total AG in total proteins was thus increased, consisting (in %) in the control animals of 42.2, during excitation – 47.4, during convulsion period of 60.3 to 70.6.

In the water-soluble cerebral proteins of rats during development of oxygen poisoning, the dynamics of the content of the firmly-bound AG were similar to the labile AG and reproduced the dynamics of total number of AG (Fig. 3). Strong convulsions resulted in a pronounced increase in the number of the firmly-bound AG (on the average up to 78 μg NH_3-N/mg N protein). The calculation of the relation of the firmly-bound AG to their total number in water-soluble rat cerebral protein (in % of control) produced the following results:

Control	Moderate excitation	Strong excitation	Convulsions
62.6	59.2	55.0	59.5

Thus, in the total rat cerebral proteins in the process of development of oxygen poisoning, which outwardly was evidenced by the increased excitation of the animal (leading to strong convulsions), we have observed an insignificant increase in the total number of AG and a change in the relationship between the labile and firmly-bound AG in the protein molecule. The ratio of labile AG/firmly-bound AG in the cerebral proteins of control animals was lowered from 1.37 to 0.42 when strong convulsion ensued. This occurred due to the increase in the number of firmly-bound AG.

In the water-soluble cerebral proteins during oxygen intoxication the number of firmly-bound and labile AG changed in parallel, and thus their relation of one to another in the process of development of oxygen poisoning remains almost unchanged.

AG of rat cerebral proteins under the action of oxygen pressure of 3.5 atm. Examination of the results concerning the action of oxygen pressure of 6 atm has revealed that the ranges of variations in all fractions of the AG of cerebral proteins of control animals and after strong convulsions were considerably lower than in the groups of animals showing different stages of excitation. It could be supposed that the dynamics of the AG number of cerebral proteins reflect to a certain degree the functional state of the animal. In order to check this supposition, we have conducted a series of experiments on the effects of oxygen pressure of 3.5 atm. At this pressure the picture of oxygen poisoning is extended. Convulsions begin at 90-120 min after the beginning of oxygen action. Prior to this one can observe various, extended in time, stages of excitation which alternate with a dream-like state. In the table are presented results of the determination of the total number of AG, labile and firmly-bound AG of water-soluble cerebral proteins of rats acted upon by oxygen pressure of 3.5 atm.

From the table it becomes evident that following the same exposure, depending on individual peculiarities one can observe different functional states of the animal. It can be also observed that there was a determined dependency between the functional state and the dynamics of AG in the water-soluble cerebral proteins. Thus, after 60 min exposure some animals were in a constant state of strong excitation, and in others slight excitation was replaced by a dreamlike state. In the last case the sum of AG of the proteins was always lower than in strongly excited animals. Especially pronounced was the change-over in the relation between the firmly-bound and the labile AG. At the time of excitation and dreamlike state the number of labile AG in the protein decreased at the same time the number of firmly-bound AG remained almost unchanged. In strongly excited animals the number of labile AG increased. These data are in agreement with results obtained with oxygen pressures of 6 atm.

AG of water-soluble cerebral proteins of rats under narcosis. Vladimirova [5], Richter and Dawson [4] and many other investigators have shown that in animals under narcosis, in opposition to what takes place during excitation, there was a decrease in free brain ammonia. The preceeding should reflect itself in the dynamics of the number of AG in cerebral proteins. We have investigated the AG dynamics of the water-soluble cerebral proteins of rats under short term ether narcosis, which according to Pertzova [8] does not noticeably change the free brain ammonia content.

From Fig. 4 it is evident that short term ether narcosis does not change the AG sum of the water-soluble cerebral proteins (control – 85.6, narcosis – 84.5 μg NH_3-N/mg N protein). However, narcosis noticeably changed the relationship between the labile and firmly-bound AG – the number of labile groups increased and the number of firmly-bound AG decreased.

DISCUSSION OF RESULTS

The obtained results attest to the intimate connection between the functional state of the animal and the

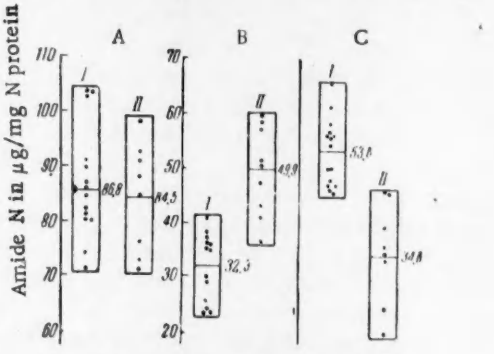

Fig. 4. Changes in the number of AG of water-soluble rat cerebral proteins following ether narcosis. A – Total number of AG; B – labile AG; V – firmly-bound AG. I – Control; II – narcosis.

AG of Water-Soluble Cerebral Proteins of Rats Acted upon by Oxygen Pressure of 3.5 atm (μg NH_3-N/mg Nprotein)

Condition of the animal	Exposure in min	Sum of AG	Labile of AG	Firmly-bound AG	Labile / Firmly-bound AG
Light excitation, from 40 min – dreamlike state	60	53.6	25.2	28.4	0.88
Light excitation, from 38 min – dreamlike state	60	56.4	26.1	30.3	0.86
Light excitation, from 30 min – dreamlike state	60	49.3	16.5	32.8	0.49
Light excitation, from 30 min – dreamlike state	60	46.6	14.2	32.4	0.44
Light excitation, from 20 min – dreamlike state	30	59.1	15.6	43.5	0.36
Light excitation, from 20 min – dreamlike state	30	54.5	8.9	45.6	0.19
Till 20 min strong excitation, from 40 min dreamlike state	60	27.1	13.3	10.8	0.96
Till 20 min strong excitation, from 40 min dreamlike state	60	20.6	7.1	13.5	0.52
Till 15 min strong excitation, from 36 min dreamlike state	60	35.5	10.0	25.5	0.40
Very strong excitation for the whole period	60	82.2	49.4	32.8	1.50
Strong excitation for the whole period	60	61.0	37.2	23.8	1.56
Very strong excitation for the whole period	60	67.5	36.2	31.3	1.17
Strong excitation	30	51.4	35.7	15.7	2.32
Strong excitation	30	45.0	34.3	10.7	3.20

dynamics of the amide and carboxyl groups of proteins. We cannot fully ascertain to what concrete state is one or another content of the amide and carboxyl groups in cerebral proteins related.

Analyzing the biological importance of the reaction between ammonia and proteins, one can assume that this is the simplest way of realizing the physicochemical possibilities of the protein molecule. Depending on the relation between carboxyl and amide groups, there is a change in the charge, and subsequently, the configuration of the protein molecule. The latter, without doubt, influences the function of the organ or tissue in which the process is taking place. We propose, that the reaction between ammonia and protein carboxyl groups is the most primitive and simple protein reaction mechanism influencing outer actions. In any, even the simplest organism, the inner medium contains a system consisting of protein, amino acids, and ammonia, which constantly interact with one another.

Observation of the mechanism of urea synthesis in mammals allows us to make the conclusion that during this process amidation and deamidation of two-carbon amino acids takes place and that this is an important link in the process, which becomes progressively more complex in the evolutionary process.

AG in a number of other cases serve as the transport form or as a reserve of ammonia that is necessary for various metabolic reactions.

Release and tie-up of ammonia in the brain is evidently a complex and a multistep process. Participation of proteinaceous substances makes this process especially significant for brain function.

From our experimental data it is evident that within the limits of the protein fraction there can be found AG of two types; quite movable, easily cleaved and the firmly-bound ones. It is fully natural that the AG of both types are formed from free carboxyl groups that do not participate in peptide linkages. It is possible to think that delegation of the AG to one type or another is determined by their spacing on the protein molecule, on the ends or in the middle, and possibly by the character of the carboxyl group radical.

SUMMARY

Different disruptions of the neural system function, brought forth by increased oxygen pressure, change the relation between labile and firmly-bound amide groups. In the total cerebral proteins of rats in a state of excitation there was observed an increase in the number of amide groups and a corresponding decrease in carboxyl groups. The change in the relation between labile and firmly-bound amide groups attests to the pronounced changes in protein molecular structure during oxygen intoxication.

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THE QUANTITATIVE RELATIONS BETWEEN PROTEIN AND NUCLEOPROTEIN FRACTIONS OF VARIOUS TISSUES

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In our previous publications [1-4] histochemical and biochemical methods have shown that the globulin fraction of cellular nuclei, obtained by 0.14 M NaCl, corresponds to the cellular juice; the nucleoprotein fraction obtained by 1-2 M NaCl – desoxyribosenucleoprotein chromation (chromosome) and acid protein, soluble in 0.05 N KOH – corresponds to the nucleolar material and residual chromosomal material. In one of the publications there was described a method for isolation of cell nuclei, which allowed preservation of a number of native characteristics [2]. The quantitative relationship among nuclear fractions of nuclei isolated by this method was determined only for the rat liver [2, 3]. The distribution of nuclear nitrogen was as follows: globulin fractions ~20%, desoxyribonucleoprotein (DNP) ~70%, acid protein ~6%, and the residual protein ~4%. The globulin fraction contained 2.6-8% RNA, DNP consisted of DNP histone and non-histone protein; the acid protein was rich in RNA (16-18%). This result differs from previous results, obtained from nuclei isolated in citrate media [5-7].

The present work was undertaken in order to clarify the problem whether the general distribution scheme of nuclear fractions of various tissues is preserved and whether there is a correlation between the fraction ratio and the morphological structure of nuclei from various tissues. The last question arose in connection with the observations of the previous work [1], where it was shown that in various mammalian tissues the determined protein and nucleoprotein fractions correspond to definite cytological structures.

METHODS

The following tissues served as materials for isolation and fractionation of nuclei: the liver, spleen and brain of the rat, chicken liver, and liver from 19-day chicken embryo, whole 11-day chicken embryo, and spermatozoa from the Azov Black Sea sturgeon (Acipenser guldenshadtii colchisus v. Marti). In order to isolate nuclei in numbers sufficient for analysis, the previously described method [2] was modified. The tissue (30-60 g) was sliced and to it there was added a mixture of solutions of sodium glycerolphosphate (0.05 M) – sucrose (of sp. gr. 1.273; pH 6.6) in a ratio of 2.5-3.5 to 1 (volume to weight). Homogenization was accomplished by a two-step passage through a bacterial mill [2], or by the cylindrical homogenization of Potter and Elvehjem with a teflon pestle. The homogenate was filtrated once through a double layer of cheesecloth and was centrifuged at 14,000 × g for 20 min, slower speeds can also be util-

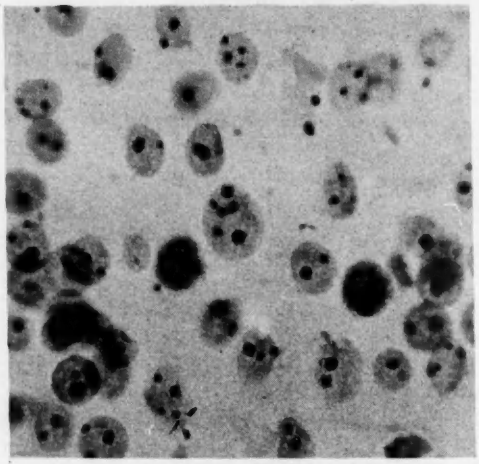

Fig. 1. Rat liver nuclei, isolated by the two-step sucrose-glycerolphosphate method. Magnification 100 × 10. Methylene blue stain.

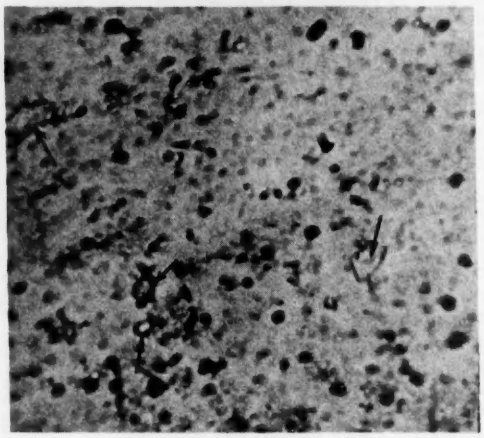

Fig. 2. Material following extraction of rat liver nuclei with 1.5 M NaCl. Together with numerous nucleoli and residual chromosomes, there can be observed strongly refractive, unstained nuclear membranes (shown with arrows). Magnification 100 × 10. Pyronin-methyl green stain.

ized (6000 × g for 45 min). A large proportion of cytoplasmic particles and whole cells remained floating in the supernatant and were discarded. The nuclei were found in a small precipitate on the bottom of the flask. At this point the nuclei are contaminated by cytoplasmic particles and hemoglobin. For this reason the nuclear precipitate was once again suspended in one half of the previous volume of sodium sucrose-glycerolphosphate (sp. gr. 1.273) with the help of the Potter-Elvehjem homogenizer. The mixture was centrifuged for 30 min at 14,000 × g or for 60 min at 7000 × g and at this time the precipitate contained pure nuclei with only a slight admixture of connective tissue fibers. Thus, by this method one can obtain at once pure nuclei from 40-60 g of tissue (Fig. 1). The nuclei gave a positive test for gellation according to Dounce [8].

The described method was utilized for isolation of nuclei from the liver, spleen, and brain of the rat, chicken liver, and embryo. In all cases there were obtained clean, morphologically complete nuclei. The nuclei of chicken tissues are of smaller dimensions and require extension of the time of centrifugation at 14,000 × g was extended to 45 min during the first run and to 60 min during the second. While working with the rat liver the volume was doubled because the spleen nuclear fraction was considerably greater than the one from the liver. The average nuclear yield was 60-70%.

The merits of the above described procedure for isolation of nuclei are that it allows work with large amounts of material and that precipitation of the nuclei is accomplished with relatively low centrifugal force.

The isolation of pure Ehrlich carcinoma nuclei by the described method was not accomplished. Even after a six-fold transfer through a bacterial mill the perinuclear cytoplasmic layer was retained. For the purpose of removal of this layer the nuclei were worked over in the Waring blendor with 0.05 M sodium citrate (pH 6.9) and after centrifugation the cytoplasm-free nuclear material was obtained. However, the nuclear integrity was destroyed and the globulin fraction, extractable by citrate, was lost.

Sperm heads were isolated by the above described method. Examination under the phase-contrast microscope did not reveal any tails after the described treatment.

Fractionation of the isolated nuclei was conducted according to the previously described method [2] with the following exceptions: The globulin fraction was extracted not twice but three times, and for the isolation of the DNP fraction the nuclei were first suspended in 0.14 M NaCl, and then to it there was added an equal volume of 2.86 M NaCl, the final concentration was adjusted to 1.5 M. As a result of this the extraction of DNP was much more rapid. Fractionation of the material during extraction was conducted in a Potter homogenizer with a teflon pestel, this excluded the possibility of contamination with glass. Analytical methods of analysis of the relation and composition of the fractions were described in the previous publication.

RESULTS AND DISCUSSION

The cytological picture of the smears of the material obtained after extraction of nuclei from various tissues, corresponds to the previously described picture for rat liver. The removal of the globulin fraction left intact all of the chromatin DNP and nucleolar RNA and the residual chromosomes. Removal of RNP with 1.5 N NaCl did not effect the nucleolar material and the residual chromosomes. Nucleoli from the brain and from Ehrlich's carcinoma appeared especially good. Extraction of the acid protein was accompanied by the disappearance of the nucleoli and the residual chromosomes. In this fashion it was always possible to notice the relation of the isolated fraction to a corresponding nuclear structure.

In addition to the previous observations [2, 3] we were able to follow the fate of nuclear walls because glass was absent from the preparations, and present when glass homogenizer of Potter was employed. In smears obtained after removal of the globulin fraction and DNP, together with the nucleoli and residual chromosomes there could be identified a large number of nonstained, strongly refractive formations.

In Fig. 2 these formations appear separate from nucleoli and residual chromosomes. However, in the extraction process with 0.14 M NaCl and during the early stages of the following 1.5 M NaCl extraction it could be observed that these formations correspond to nuclear walls which break and wrinkle in the extraction process. Sometimes there could be seen within them nucleoli which were subsequently washed out with sticky solution of DNP.

Following alkaline extraction the walls were preserved, however in connection with their swelling their structure was less prominent. In this way our investigation of nuclei free of cytoplasmic contaminants support the view that the residual proteins was material from nuclear walls. This is in agreement with data obtained by Soudek and Benes on nuclei isolated in citrate media [9].

Data on the protein and nucleoprotein composition of the isolated nuclei are shown in the table.

In the table, besides the results of the present study, are presented data for the rat liver which were corrected because in the previous study the values for DNA were lower and the values for DNP correspondingly higher, due to the fact that the purchased DNA used as the standard was impure. Subsequently pure DNA was obtained by a method worked out by one of the authors [10]. Using this preparation of DNA as the standard we recalculated the values for the rat liver. In addition, during the calculations, the cross-contamination of the DNP-fraction and acid protein fraction was taken into account [2].

As can be seen from the table, the distribution of material among nuclear fractions of various tissues was of

Proteins Fractions of Nuclei from Various Tissues (mean percent content of the fractions the the ratio acid protein/DNA

Fractions	Rat			Mouse	Chicken			Sturgeon
	liver	spleen	brain	ascites cells Ehrlich's carcinoma	liver	embryonic liver**	whole*** chicken embryo	spermatozoa
	number of experiments							
	5	1	2	2	4	1	2	3
Globulin	20.4	26.2	17.0	—*	29.0	31.6	25.0	3.0
DNP	67.6	64.3	66.1	66.6	52.6	47.0	58.6	90.5
DNA	22.2	25.2	19.3	18.2	19.4	17.8	15.4	42.2
Nonhistone protein	12.4	5.2	13.8	9.8	6.6	6.2	16.6	2.6
Histone and protamine	33.0	35.9	33.0	38.6	26.6	23.0	26.6	45.7
Acid protein	7.5	5.5	13.1	30.0	12.1	16.2	11.5	4.1
Residual protein	4.5	4.0	3.8	3.4	6.3	5.2	4.9	2.4
Acid protein/DNA	0.34	0.22	0.68	1.65	0.62	0.91	0.75	0.097

* Calculation conducted on the nuclear proteins and nucleoproteins without account for the globulin fraction which was not determined.

** Embryo 19 days old.

*** Embryo 11 days old.

the same order. The distribution of nuclear nitrogen was of the following order: globulin fraction 20-30%, DNP about 65%, acid protein from 5 to 15%, and the residual protein about 5%. The largest variation within nuclear material occurred in the acid protein, i. e., the nucleolar and residual chromosome material.

If the quantity of DNA in one nucleus of cells of a given tissue is practically constant, then the ratio acid protein/DNA can characterize the amount of nucleolar material in the nucleus. As can be seen in the table this ratio is lowest for sturgeon sperm heads; in the rat brain, it is higher than in the liver, and in the liver higher than in the spleen; this corresponds to the cytological picture of the nuclei from these tissues (the nucleolar apparatus in the brain is more developed than in the liver and the spleen). Even higher was this ratio in Ehrlich's carcinoma, where the nucleolar apparatus is very well developed. The acid protein fraction of all of the tissues was rich in RNA; the acid protein of rat spleen contains 17% RNA, rat brain-22% RNA.

In the DNP fraction approximately one half of the nitrogen falls into histone. The content of non-histone protein varies in different tissues. Thus, in rat liver and brain it was considerably higher than in the spleen. The highest content of non-histone protein was observed in nuclei of 11-day old chicken embryo; in chicken liver it was about three times lower. However, insufficient quantity of the material prevents concrete conclusions concerning specific distribution of non-histone protein. Thus, the nuclei of all of the investigated objects are constructed according to a common type [2, 3]. At the same time nuclei of various tissues differ in their content of acid protein, and the content of acid protein (ratio of acid protein/DNA) correlates with the development of the nucleolar apparatus.

Special attention is due the question concerning the protein composition of nuclei from malignant tissues. Our attempts to obtain pure nuclei by the above described method and by other methods (utilization of glycerine instead of sucrose, addition of palmitin-sulfate etc.) from majority of malignant tissues (sarcoma M., ascites hepatoma, sarcoma of Tarashchinska, Jensen's sarcoma, Walker's rat carcinoma, rabbit malignancy of Brown-Pierce) did not give satisfactory results. In all cases the nucleus was surrounded by a cytoplasmic layer. This on one hand made the DNP extraction difficult, and on the other hand it gave material falling into the acid and residual protein fractions, as a result of which their percent content sharply increased. Evidently fractionation of malignancy nuclei, containing the perinuclear layer, by the above described method produced results practically the same as the ones obtained in our laboratory by the citrate method [11, 12]. Thus after fractionation of nuclei from Walker's rat carcinoma the DNP fraction was 22%, acid protein – 59%, and residual protein – 19%.

In case of Ehrlich's carcinoma the perinuclear material was removed during extraction of the globulin fraction. When the globulin fraction of nuclei from ascites cells was not taken into account then the acid protein fraction content was very high, which reflected the development of the nucleolar apparatus and is, evidently, characteristic for malignancies in general.

Remembering, that according to Saidov's data [12] in nuclei obtained by the citrate method the acid protein content was high from the first few days after inoculation and the residual protein accumulated stepwise, it became evident that it is difficult to compare ascites cells with cells from solid malignancies. Likewise, cytological analysis showed that utilization of various malignancies yielded isolated nuclei that were always surrounded by a perinuclear layer, which was never evident in normal tissues.

On this basis it could be concluded that the presence of perinuclear layer is characteristic of malignant nuclei. Fractionation of such nuclei showed presence in them of a large amount of insoluble fraction of residual protein [11, 12]. Thus, it is possible that differences in the composition of malignant nuclei are connected first of all with the peculiarities of the nuclear envelope, perinuclear layer and the nuclear-cytoplasmic relations in malignancies.

In connection with this a great amount of interest is centered around the question of preservation in the malignant nuclei, after their isolation, of the perinuclear zone, its nature and composition and its relation to residual and acid proteins. These questions are the subject of further study in our laboratory.

Finally, we have studied the relation of protein and nucleoprotein fractions in the sturgeon spermatozoa. As is shown in the table, in the nuclei, or more correctly in the sperm heads, we observed only 4% of acid protein. It should be brought to attention that in difference to acid proteins acid protein of sperm heads contained only 2.5% RNA. Since there are no nucleoli in sperm heads, then the presented figures reflect the content of residual chromosomes. Other non-basic proteins in spermatozoa were also found in a very small quantity. The very small content of non-histone proteins in sperm nuclei evidently reflects the lack of synthetic activity in them. This condition supports the supposition that DNA in a complex with protamine is found in an inert like, non-active state [13, 14].

SUMMARY

A modification of the sucrose-glycerophosphate method for isolation of a large number of nuclei from various mammalian organs is described.

A study was made of the distribution of nuclear material between protein and nucleoprotein fractions and the content of the latter in nuclei of rat spleen, liver, and brain, Ehrlich ascites carcinoma of mice, chicken liver and embryo and sturgeon sperm heads. The general character of the composition of the nuclei of all of these tissues was similar. The most striking difference in the composition of nuclear material consisted in the percentage of the acid protein fraction which represented the material of nucleoli and of residual chromosomes. The ratio acid protein:DNA is correlated with the development of nucleolar apparatus.

From the majority of experimental malignancies pure nuclei could not be obtained. They retained the perinuclear-cytoplasmic layer which attests to the alteration in the relation between the nucleus and cytoplasm in malignancies. It is suggested that proteins of this layer during fractionation fall into the residual and acid protein fractions. Sperm heads contain small amounts of acid proteins and other non-histone proteins. This is most likely related to their low synthetic activity.

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N-DIHYDROXYETHYLGLYCINE AS A BUFFER IN BIOCHEMICAL WORK

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The continually expanding scope of biochemical practice creates a constant demand for new compounds possessing buffering properties.

Comparatively recently workers in most fields of biochemistry used inorganic buffering mixtures, only rarely turning to some organic compounds with buffering properties. Yet, of the few existing mineral buffers, only four are actually acceptable for biochemical work; they are, phosphate, borate, carbonate and ammonium buffers. The inconvenience of the two latter ones mentioned is due mainly to their instability, but the two former buffers also have their disadvantages.

Thus, phosphate is incompatible with Ca^{++}, and poorly compatible with Mg^{++}. Phosphate buffer cannot be used in systems where reaction progress is followed by the change in phosphate content. Borate buffers are also unsuitable in a number of systems. Insufficient attention has been paid in the past to the fact that borate buffers are not as inert to some active biological compounds as would appear from superficial consideration.

The ease with which certain hydroxylic compounds react with boric acid is well known. Of the biologically active compounds in this group, the following may be quoted:

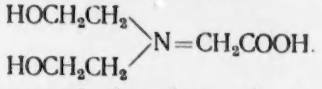
1. Adrenaline base has a very low solubility in water or in phosphate buffers at neutral or weakly alkaline pH, but dissolves well in borate buffers under the same conditions [1], which indicates some complex formation with the borate ion.

2. Phosphates of sugars with similar structures are very difficult to separate chromatographically. The separation may be made much more effective if carried out in borate buffer, a phenomenon due to the ability of the borate ion to form complexes with the sugars containing hydroxyl groups in the cis-configuration [2-4].

The organic buffers, as distinct from the mineral ones, are far more numerous and varied and, although the larger part of those were found to be unsuitable for biological work because of low solubility or insufficient inertness towards the biological systems, individual compounds were found to be very valuable as buffers and have found wide application.

In the recent years trimethylolaminomethane, often referred-to as "TRIS", which was suggested by Gomori [5], has become well known. Trismethylolaminomethane, sometimes referred to (in the American literature) as trishydroxymethylaminomethane, is prepared by the condensation of nitromethane with formaldehyde, with the subsequent reduction of the trimethylolnitromethane. The last step is difficult to carry out in a general biochemical laboratory, which restricts the availability of Tris buffer.

The latter circumstance prompted us to suggest as a base for the preparation of buffer solutions with pH within the biological limits, the compound N,N-di-(2-hydroxyethyl)-aminoacetic acid, of the following structure:



This compound, whose shorter, though somewhat inaccurate, name is dihydroxyethylglycine, may be easily obtained from monochloroacetic acid and diethanolamine according to the reaction:

$$(HOC_2H_4)_2NH + ClCH_2COONa \rightarrow$$
$$\rightarrow (HOC_2H_4)_2NCH_2COOH + NaCl.$$

The necessary reactants are readily available and the synthesis may be carried out in any biochemical laboratory according to a method previously published in detail [6].

Chemical Characteristics of Dihydroxyethylglycine

Dihydroxyethylglycine is a white crystalline powder, it is non-hygroscopic and may be stored indefinitely at room temperature. It may be brought to any desired degree of purity by recrystallization from 80% methanol. Its melting point (with decomposition) is 190-192%.*

The buffering properties of hydroxyethylglycine are very similar to those of Tris, but the maximal buffering capacity of the new buffer lies in the more alkaline range of pH 8.3-8.4, while for Tris this range is pH 8.0-8.15. While in this respect the new compound is possibly inferior to Tris, it has the advantage of being completely inert towards compounds containing the carboxylic group and others, such as aldehydes and quinones, while Tris is known to be reactive with such compounds.

* Since the melting point of the compound found is considerably lower if the capillary tube with the sample is heated for any length of time, the apparatus should be preheated to 185-190° before inserting the sample.

The chemical inertness of dihydroxyethylglycine is, understandably, also limited. Like many other hydroxylic organic compounds, including, to a certain extent also Tris, dihydroxyethylglycine is sensitive to active oxidizing agents. For this reason, for instance, the quantitative determination of glucose according to Hagedorn-Jensen, which involves heating the sample in alkaline potassium ferricyanide solution, cannot be successfully carried out in the presence of comparatively high concentrations of dihydroxyethylglycine.

Buffer solutions of desired pH are prepared from a stock solution of dihydroxyethylglycine by the addition of the required amount of NaOH or KOH. Approximate values of the ratio of amino acid to alkali for given values of pH may be obtained from the data of potentiometric titrations of 40 ml of 0.1 M DEG (dihydroxyethylglycine) with a 0.1 N solution of NaOH (Table 1). The data also indicate that the temperature coefficient of DEG buffer solutions is low (less than 0.2 pH units in a temperature change of 17°).

The Biological Inertness of Dihydroxyethylglycine

The chief advantage of the buffer recommended above is it inertness to many enzymes. Quoted below are some comparative data on a number of enzymatic reactions carried out in DEG buffer and in other conventionally employed buffers.

Activity assay of 6-phosphofructokinase.† The enzyme was assayed according to Neifakh et al., [7]. The results are shown in Table 2.

Activity assay of baker's yeast hexokinase.‡ The enzyme was assayed according to a published method [8] utilizing the decrease in easily hydrolysed phosphorus of ATP. The results are shown in Table 3.

The effect of DEG on the enzymatic synthesis of Coenzyme A.** The assay was carried out as follows: Rat brain was homogenized with ten volumes of the solution containing: buffer, pH 7.5 (0.055), ATP (0.005), calcium panthotenate (0.0016), cysteine (0.016), $MgCl_2$ (0.03) and versene (0.01).

TABLE 1. Potentiometric Titration of a DEG Solution with 0.1 N NaOH

ml 0.1 N NaOH added to 40 ml 0.1 N DEG		pH	
ml	moles % of DEG	at 20°	at 37°
0.0	0	5.1	–
2.0	5.0	7.0	–
3.0	7.5	7.15	–
4.0	10.0	7.35	–
5.0	12.5	7.5	–
6.0	15.0	7.6	–
7.0	17.5	7.65	–
9.0	22.5	7.8	–
10.0	25.0	7.85	–
11.0	27.5	7.95	–
12.0	30.0	8.0	7.8
15.0	37.5	8.1	7.95
17.0	42.5	8.2	8.05
19.0	47.5	8.3	8.1
20.0	50.0	8.35	8.15
23.0	57.5	8.45	8.30

When more concentrated solutions (0.5-1.0 M) of the buffer are required, the composition may be calculated according to the formula:

$$\log\left(\frac{100\ M}{N \times V} - 1\right) = 8.25 - pH,$$

where M = molarity of the final buffer solution, N = the normality, and V = the volume, of the alkali solution to be added to the suspension of dihydroxyethylglycine for the preparation of 100 ml of the buffer solution of desired pH.

Parallel incubations in the various buffers were carried out over various time intervals, and at the end of the incubation period CoA was assayed according to Kaplan and Lipmann [9], with the modification of Sytinskaya [10]. Coenzyme A content was assayed as level of p-aminobenzoate acetylation and expressed as n-acetyl-p-aminobenzoate per unit weight of brain tissue taken (in mg%). The results are shown in Table 4.

** This part of the work was carried out by K. G. Gromova.
† This part of the work was carried out by M. P. Fomina.
‡ This part of the work was carried out by G. V. Titova.

TABLE 2. Activity Determination with 6-Phosphofructokinase in DEG Buffer. Thereaction mixture contained: $MgCl_2$ (0.005), ATP (0.0025), F-6-P (0.004), buffer, pH 7.4 (0.04), enzyme. Incubation time – 15 min

P-transfer from ATP to F-6-P, μg	
in borate buffer	in DEG buffer
20.8	24.0
22.4	24.0
17.5	19.8
27.0	31.8

Here, as in all other cases, all concentrations showed are final molar concentrations.

TABLE 3. The Determination of Hexokinase Activity in DEG Buffer. The reaction mixture contained: $MgCl_2$ (0.006), glucose (0.0015), ATP (0.0012), buffer, pH 7.9 (0.025) and enzyme. Incubation time, 15 min

P-transfer from ATP to glucose μg/ml enzyme solution	
in borate buffer	in DEG buffer
200	200
230	230
120	130

TABLE 4. Enzymatic Synthesis of Coenzyme A (acetylated p-aminobenzoic acid, mg% on weight of brain tissue used) Means of 5-6 Experiments

Buffer	Before incubation	Incubation time, min		
		30	60	90
Glycylglycine	22.5	27.5	31.0	30.5
DEG	25.0	30.5	31.5	28.5

TABLE 5. Oxidative Phosphorylation in Cellular Extracts. The reaction mixture contained: $MgCl_2$ (0.006), KF (0.0s), glucose (0.04), ATP (0.001), phosphate buffer, pH 7.6 (0.006), DEG buffer, pH 7.6

Final DEG conc., M	Uptake, per ml of extract		P : O
	O_2, μ atoms	P, μmoles	
–	10.4	26.9	2.5
0.006	10.7	27.2	2.5
–	7.6	23.4	3.0
0.006	7.5	22.5	3.0
0.006	8.0	23.4	2.9
–	6.4	14.2	2.2
0.012	6.2	14.5	2.3

TABLE 6. Oxidative Phosphorylation in Mitochondria. The reaction mixture contained: $MgCl_2$ (0.003), KF (0.02), glucose (0.04), ATP (0.001), glutamate (0.004), phosphate buffer, pH 7.4 (0.004), DEG buffer, pH 7.4

Final DEG conc., M	Uptake, per 15 mg of mitochondrial protein		P : O
	O_2, μatoms	P, μmoles	
–	2.4	4.2	1.7*
0.006	2.8	4.2	1.5*
–	2.1	6.3	3.0
0.004	2.0	6.4	3.2
0.004	1.9	5.6	2.9
–	3.1	7.2	2.3
0.004	3.2	7.2	2.2
–	2.4	7.4	3.0
0.008	2.4	7.5	3.1

*Experiments carried out with a suspension of large particles. Unlike the remaining experiments, the reaction mixture here contained $MgCl_2$ (0.012) and phosphate buffer (0.012).

Oxidative phosphorylation.†† The experiments were carried out with crude extracts, large particle suspensions and pure mitochondria prepared from guinea pig livers. The reaction mixture was incubated in Warburg vessels for 20 min. The gas phase was air. Oxygen uptake and inorganic P uptake were determined and the P/O ratio calculated. The results are shown in Tables 5 and 6. Apart from these experiments, DEG buffer was also successfully used instead of Tris buffer in the reaction mixture with the Hoffman-Berling [11] models for the reproduction of motility of infursorial flagella.‡‡

SUMMARY

N,N-di(2-hydroxyethyl)-aminoacetic acid (dihydroxyethylglycine) was shown to be a suitable buffer for biochemical work, being sufficiently inert biologically.

The inertness of dihydroxyethylglycine to various enzymes was demonstrated on the example of the respiratory chain enzymes, yeast hexokinase, muscle phosphofructokinase, the enzymatic system synthesizing Coenzyme A and the Hofmann-Berling models for the reproduction of motility in infursorium flagella.

Dihydroxyethylglycine can be easily synthesized from readily available starting materials according to a published procedure, and it can be used as a substitute for the less readily available trishydroxymethylaminomethane (Tris) buffer.

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†† This part of the work was carried out by L. I. Niselovskaya.

‡‡ The experiments were carried out by V. Ya. Aleksandrov and N. I. Aronet, of the Botanical Insitute, USSR Academy of Science, Leningrad.

••• Original Russian pagination. See C. B. translation.

THE EFFECT OF ASCORBIC ACID AND BIOFLAVINOIDS ON THE ACTIVITY OF HYALURONIDASE

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Among the biological problems which have a direct relation to the physiology and pathology of the living organism, the question of tissue and vascular permeability is of great interest and is still far from being settled. A large amount of information indicates the participation of enzymatic processes in the regulation of permeability. An important role is played particularly by hyaluronidase, whose action is directed to the mucopolysaccharides of connective tissue structures (hyaluronic acid, chondroitin sulfates A and C). The aggregate state of hyaluronic acid, a constituent part of the main intercellular substance, and the nature of its bonds with protein largely determine the conditions of intercellular exchange, barrier function, and reparative and involutional processes which occur in the organism. The destruction of these bonds and depolymerization and hydrolysis of hyaluronic acid caused by hyaluronidase increase the permeability of tissues and capillaries and change the rate of lymph circulation in the tissue spaces. In this connection it is very interesting to study hyaluronidase inhibitors which may have practical use in some diseases in the clinic.

Vogt and Szent-Györgyi [1,2] showed the favorable effect of ascorbic acid and vitamin P (citrin) on damage to capillary permeability. After the appearance of the work of Beiler and Martin [3] it was suggested that the action of ascorbic acid and vitamin P on vascular permeability depended on inhibition of tissue hyaluronidase. An increase in inhibiting action of ascorbic acid was established when it combined with preparations which had vitamin P activity (rutin, esculin, hesperidin, etc.). This permitted the suggestion that the degree of activation of inhibition by ascorbic acid on hyaluronidase in the test tube could serve as a criterion of attachment of the compound to a group on vitamin P [4]. The inhibition of hyaluronidase activity by ascorbic acid was shown in further experiments on rabbits using a skin test [5], and the action of vitamin P was shown in the work of Levitan [6]. Later a number of authors showed by animal experiments using isolated tissue membranes that still stronger inhibition of the effect on hyaluronidase was possessed by phosphorylated derivatives of vitamin P analogs, especially hesperidin [7, 8]. Thus, everything confirmed the fact that insufficient introduction of ascorbic acid and bioflavinoids into the organism could cause increased activity of hyaluronidase in the tissues and as a result, increased permeability [9]. However, such a viewpoint contradicts the solidly established fact of the accumulation of viscous mucopolysaccharides, including hyaluronic acid, in the connective tissues in scurvy [10], which indicates the absence of activation of tissue hyaluronidase. There have been indications that ascorbic acid does not affect vascular fragility and that a connection cannot be established between a change in capillary permeability and the resistance of the human organism due to ascorbic acid [11]. Elster [12] could not show an inhibition by the action of rutin on a purified preparation of hyaluronidase in experiments on white rats, and Rodney and coworkers [13] noted a nonspecific effect of flavone derivatives on hyaluronidase.

The contradictory data on the reactions of hyaluronidase-hyaluronic acid with ascorbic acid and bioflavinoids led us to carry out a study of their antihyaluronidase activity. The investigation was carried out by the use of two methods for determining hyaluronidase activity: viscosimetric, as the most direct way to estimate the degree of destruction of hyaluronic acid, which agrees well with the results of biological study, and turbidimetric, used in most other work.

METHODS

Preparations

Hyaluronidase was obtained from beef testes by the method of Dorfman with use of the method which gives the maximum yield of the enzyme [14, 15]. The enzyme preparation obtained by this method can be kept for three years without appreciable loss in activity. The process of purification can be interrupted at any stage, and a satisfactory degree of purity can be obtained even after the first salting out and dialysis. Actually we carried out a double fractionation of the testis extract with ammonium sulfate followed by dialysis and lyophilic drying of the dialyzate to obtain light white flakes of hyaluronidase. The activity of the hyaluronidase with strict maintainance of pH and temperature (2-4°) was satisfactory in the whole process of isolating the enzyme. Completeness of dialysis was controlled by measuring the electrical conductivity or intensity of color of the wash water with the Nessler reagent.

Preparations were kept in a vacuum desiccator with $CaCl_2$ or in a paraffined flask in the refrigerator. The activity of the separate samples of hyaluronidase was 700-800 viscosimetric units/mg of N. The N content in the preparation, determined by micro-Kjeldahl, was 13%

Hyaluronic acid was extracted from human umbilical cords by the method of Kostenko [16] and kept in a desiccator with $CaCl_2$ in the refrigerator. In all we obtained four different preparations of hyaluronic acid with varying degrees of purity, controlled by content of N and glucosamine. The N content in the preparations varied between 3.61-5.94%, and the glucosamine, between 32.4-36.6%. Glucosamine was determined by the modified method of Elson-Morgan [17]. Since acetyl acetone and p-dimethylaminobenzaldehyde had the greatest effect on the development of a nonspecific color, they were especially purified. Acetyl acetone (C.P.) was distilled at 100 mm Hg and for the study we collected the colorless fraction which boiled at 83-84.5°. The solution was kept in a dark glass vessel in the refrigerator. Rutin, esculin, tea leaf catechin, hesperidin methylchalcone • and ascorbic acid (C.P.) were kept in a cool, dark place.

Ascorbic oxidase was obtained by the method of Tauber [18] from pumpkin C. peppo condensa. The activity was detemined by titration with 2,6-dichlorophenol indophenol of the ascorbic acid which remained after the action of the enzyme. In the experiments we used a solution of ascorbic oxidase which oxidized at pH 6.4 and 37°, 1.0 mg of ascorbic acid in 120 min.

Viscosimetric Method

The basis for the determination of hyaluronidase activity was the modified method of Tolksdorf [19]. In this work we took account of the results of Alburn and Whitley [20] on the optimum pH value and salt composition of the medium for the action of hyaluronidase.

The solution of substrate was prepared by dissolving 200 mg of hyaluronic acid in 100 ml of twice distilled water. The solution was stable when kept in the refrigerator for six days. The enzyme solution was prepared in the form of an 0.08% solution (20 mg of hyaluronidase was dissolved in 25 ml of five times diluted Mcilwain citrate-phosphate buffer, pH 6.4, containing 0.09 M NaCl) and was kept without loss of activity at 2° for six days. Ascorbic acid and bioflavinoids were used as 0.4% solutions in the same buffer solution; they were kept 2-3 days in the refrigerator.

The relative viscosity of the hyaluronic acid was determined in the Ostwald viscosimeter. The supply of solution to the upper reservoir was maintained under pressure, using a special apparatus. This method prevented the formation of bubbles in the studied liquid and increased the reproducibility of the results. The enzymatic reaction was carried out in a water thermostat at 37° ± 0.05°. For this purpose, 4.0 ml of aqueous solution of hyaluronic acid was mixed with 1.0 ml of citrate-phosphate buffer and 4.5 ml of the mixture was put in the Ostwald viscosimeter, which had first been placed in the thermostat. At the same same time we prepared the working solution of hyaluronidase by tenfold dilution of the original solution of the enzyme with five times diluted citrate-phosphate buffer, and in the study of the effect of ascorbic acid

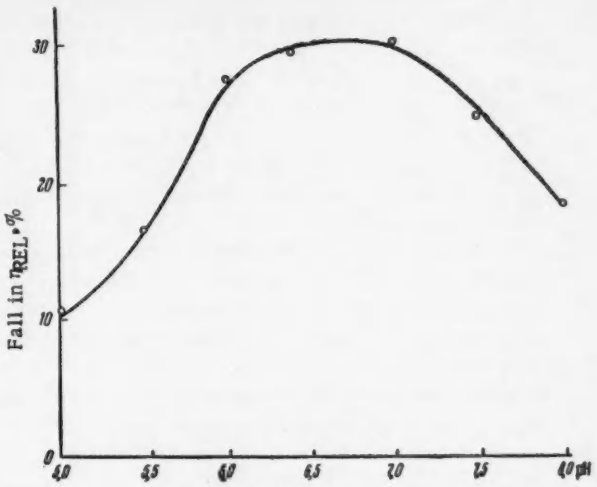

Fig. 1. Effect of pH on hyaluronidase activity. Data correspond to ten minutes from start of the reaction.

and bioflavinoids on hyaluronidase, with the buffer solution of the corresponding substance. Five ml of the working solution of hyaluronidase (with or without additions) was kept in the water thermostat for varying periods of time (not less than 15 min). After measurement of the initial time of outflow of the hyaluronic acid solution we placed in the viscosimeter 0.5 ml of working solution of hyaluronidase. During the next 30 min we made repeated measurements of the viscosity of the substrate, and the resulting data were expressed in units of relative viscosity $\eta_{rel} = t_{substrate}/t_{solvent}$, where t is the time of outflow in seconds). As the unit of enzymatic activity we took the amount of enzyme which caused a fall in relative viscosity of hyaluronic acid of 50% in 30 min. The activity of hyaluronidase (A) was calculated by the formula $A = 1/R \cdot 10^3$, where R is the time from the beginning of the enzymatic reaction in seconds during which η_{rel} falls by 50% [21]. The error in the determination was not more than 5%.

It is known that pH, salt composition of the medium, and concentration of the reacting substances can markedly change the character of the reaction of enzyme with substrate, permitting or suppressing the inhibiting action of the substances studied [22]. As a result of a series of experiments using the viscosimetric method we established that at 0.09 M NaCl in the enzyme mixture, which was chosen on the basis of the literature data [20] as the most favorable, the hyaluronidase was equally active at pH values of 6.0-7.5 (Fig. 1). More accurate determination of the optimum pH by the viscosimetric method was impossible. Hence we took as the optimum the pH value 6.4 which had been established earlier by the turbidimetric method [20].

The concentration of hyaluronic acid, as already mentioned, was established by the viscosimetric method

• The preparation was kindly supplied by M.N. Zaprometov and Prof. V.N. Bukin.

as 0.2% in the starting solution, which gave a content of 7.2 mg of hyaluronic acid in 5.0 ml of enzyme mixuture. Depending on the hyaluronic acid preparation, the initial viscosity of its buffer solution (4.5 ml at 37°) was not less than 2.15-3.5. Obvious hyaluronidase activity was found to depend directly on initial viscosity of the substrate and fell 5.7 times with decreased initial viscosity of hyaluronic acid from 3.10 to 2.16 (Table 1).

Therefore in our determinations we compared results obtained on solutions of hyaluronic acid with the same initial values of η_{rel}. This eas especially important in work with substrates whose viscosity was less than 2.90-3.00. They could not serve for determination of the absolute hyaluronidase activity, but were entirely satisfactory for comparative estimation of its action. In the experiments on determining the optimum pH and action of ascorbic acid, the hyaluronidase concentration was so selected that in 30 min there was not complete destruction of the substrate and complete loss of specific viscosity (4 μg/ml). Later we used more concentrated hyaluronidase (8 μg/ml), which permitted us to follow the dynamics of the inhibiting action of the added substances. The content of ascorbic acid and bioflavinoids was 1.0 mg per 5.0 ml of enzyme mixture.

TABLE 1

Effect of Initial Viscosity of Substrate on Hyaluronidase Activity citrate-phosphate buffer, pH 6.4; 0.09 NaCl; Hyaluronidase 8 μg/ml; volume of mixture 5.0 ml. Hyaluronidase activity expressed in viscosimetric units/mg N

Starting value of η_{REL}	Activity	Starting value of η_{REL}	Activity
3,10	685	2,60	356
3,05	650	2,54	292
3,02	710	2,49	266
2,92	640	2,48	266
2,75	460	2,44	264
2,70	458	2,16	122

Turbidimetric Method

The turbidimetric determination of hyaluronidase activity was carried out in several variants, one of which entirely corresponded with the method used in the work of Beiler and Martin [3]. The difference consisted only in the stabilization of the horse serum, which was heated on a boiling water bath at pH 3.1 for 30 min [19]. The conditions of carrying out the enzymatic reaction according to Beiler and Martin were not suitable for the action of hyaluronidase, evidently because of the absence of the activating ion Cl and the related nonoptimal pH value. [20]. The preparations of hyaluronidase and hyaluronic acid used in our experiments differed from the preparations obtained by Beiler and Martin, but were sufficiently pure. In order to avoid the possibility of side effects (contaminants) on the results of the enzymatic reactions, we ran parallel samples with use of commercial preparations of hyaluronidase and hyaluronic acid (L. Light and Co.). Also, some of the experiments were carried out under optimal conditions (pH 6.4, 0.1 M acetate buffer with 0.09 M NaCl).

Hyaluronic acid was used in the form of a 0.16% solution in 0.1 M acetate buffer, pH 6.0, the solution could be kept at 2-4° for a week. For the investigation we used hyaluronic acid preparations which had slight viscosity (η_{rel} = 1.5 in 0.2% solution at pH 6.0 in the presence of 0.09 M NaCl at 37°); this had positive value in the development of cloudiness.

Hyaluronidase in the form of a 0.1% solution was prepared in an analogous buffer solution and kept in the refrigerator; it was diluted to the desired concentration immediately before use. For each determination we took 1-3 turbidimetric units of hyaluronidase. As the unit of enzyme activity we took the amount of enzyme which caused a fall in the turbidity which develops after mixing 0.2 mg of hyaluronic acid with acidified horse serum to a value which corresponded to a content of 0.1 mg of hyaluronic acid in the sample.

As the serum protein we used acidified stabilized horse serum [19]; it was kept in the refrigerator at 2-4°; before use the serum was diluted fivefold with 0.5 M acetate buffer, pH 4.2. The solution of ascorbic acid was prepared by addition to its 0.2% aqueous solution, first brought to pH 6.0, of an equal amount of 0.2 M acetate buffer, pH 6.0. All later dilutions were carried out with 0.1 M buffer solution with the same pH value. Because of such dilutions, the pH remained unchanged, and the ionic strength in the control and experimental determinations did not change.

For carrying out the enzymatic reactions we placed in the water thermostat (37°) two test tubes, one of which contained the hyaluronidase solution (2-6 turbidimetric units/ml), and the second, a mixture of 0.5 ml (0.8 mg) of hyaluronic acid and 1.0 ml (1.0, 0.5, or 0.1 mg) of ascorbic acid. After 15 min, we added to the contents of the second tube 0.5 ml of enzyme and mixed carefully. After 30 min incubation, 0.5 ml of substrate-enzyme mixture was mixed with 2.0 ml of diluted horse serum. The intensity of the resulting turbidity, which was directly proportional to the amount of undecomposed hyaluronic acid, was measured after standing 15 min at room temperature on an FEK-N-54 microcolorimeter nephelometer in a cuvette 3 mm thick at a wave length of maximum transmission, 360 mμ (Filter No. 1). At the same time we ran a series of control determinations by the plan suggested by Beiler and Martin with consideration of the turbidity which resulted from reaction of the acidified protein with 0.2 mg and 0.1 mg of hyaluronic acid. Determination of hyaluronidase activity in the separate samples was carried out by direct comparison of the turbidity formed after action of the hyaluronidase. The experimental error did not exceed 5%.

In some of the experiments we kept the ascorbic acid first for 60 min with hyaluronidase, after which a corresponding amount of this mixture was added to the substrate.

When these conditions were maintained the method gave reproducible results, but results which varied considerably depending on the preparations of hyaluronic aicd; this can be explained by the differences in properties, degree of purity, and method of obtaining the substrate. The most reproducible results were obtained in preliminary experiments with use of unstabilized serum.

Because of the great sensitivity of the course of the enzyme reaction to the pH of the medium, we carefully controlled the pH of the buffer solutions and the enzyme mixtures in both methods. Determination of the pH was carried out with a LP-5 potentiometer with a glass electrode, by the usual method. When ascorbic acid and bioflavinoids were added to the enzyme mixture, the variation in pH from the given values was not more than 0.05.

EXPERIMENTAL

In the first series of experiments we studied the effect of ascorbic acid on the activity of hyaluronidase when it was incubated with the enzyme before beginning the reaction, by the use of the viscosimetric method. For this purpose 0.2 mg of hyaluronidase was kept for varying periods of time with 10.0 mg of ascorbic acid in a volume of 5.0 ml at pH 6.4 and 37°, from which 0.5 ml portions were added to the substrate solution. However, the results of this series of experiments (Fig. 2) indicated the absence of any effect of ascorbic acid on hyaluronidase independent of length of their contact. Preliminary keeping of the substrate with ascorbic acid also did not protect it from the action of hyaluronidase. No effect was shown as the result of change in ratio of concentrations of enzyme and substrate. Such a clear difference in these data from the facts known in the literature on the inhibitory action of asorbic acid suggests that the negative result may depend on an unfavorable pH and salt composition of the medium. This indicates the necessity of carrying out a series of experiments under conditions which correspond to the optimal [23] for the development of inhibitory effects of flavones and flavone derivatives on hyaluronidase (citrate-phosphate buffer, pH 7.0, 0.012 M NaCl). However, under these conditions ascorbic acid did not suppress hyaluronidase activity.

One of the most likely explanations of our negative results may be the suggestion that the inhibitory action of ascorbic acid in hyaluronidase which was found by Beiler and Martin [3] depends on its oxidation in the reaction mixture. Determination of the stability of ascorbic acid under the experimental conditions showed that its five hour incubation with hyaluronidase at 37° and pH 6.4 did not cause marked decrease in amount of ascorbic

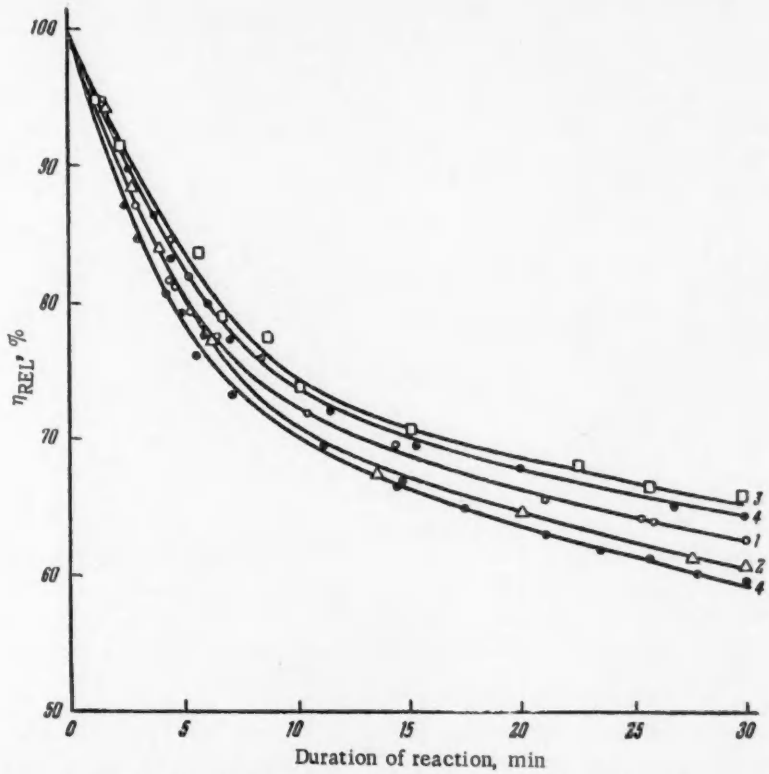

Fig. 2. Effect of incubation of hyaluronidase with acorbic acid on enzymatic activity. Preliminary incubation, min: 1) 60; 2) 180; 3) 240; 4) 240 (without ascorbic acid).

acid. In view of this, we studied the effect of oxidation products of ascorbic acid on hyaluronidase.

Hyaluronidase was incubated with ascorbic acid and ascorbic oxidase (pH 6.4), after which part of the enzyme treated in this way was added to the substrate. Further oxidation of ascorbic acid in the substrate-enzyme mixture was sharply slowed down, evidently as a result of the inhibitory effect of hyaluronic acid or misture of chondroitin sulfuric acid. Control of the completeness of oxidation of ascorbic acid was carried out by titration of aliquot parts of the enzyme mixture with 2,6-dichlorophenol indophenol in an acid medium. The results showed that neither dehydroascorbic acid nor the process of oxidation of ascorbic acid by ascorbic oxidase itself had an effect on the activity of hyaluronidase (Fig. 3). In their turn, the oxidation products and ascorbic acid scarcely changed the viscosity of hyaluronic acid, decreasing it after 30 min by not more than 8-9%. Hyaluronidase, inactivated by 30 min heating on a boiling water bath, caused a decrease in η_{rel} of hyaluronic acid of 4-5%. Such a slight difference in the control determinations only confirms the absence of an inhibitory action of ascorbic acid on hyaluronidase.

The only possible explanation of the facts, from our point of view, is the assumption that the action of ascorbic acid on hyaluronidase is very slight and can be produced only when it is combined with some activating agent, especially the bioflavinoids. Therefore we studied the effect on hyaluronidase of rutin, esculin, and hesperidin methylchalcone in the presence of ascorbic acid and without it. To 5.0 ml of the enzyme mixture we added, besides the substrate (7.2 mg), 40 μg of hyaluronidase and, per 1.0 mg of ascorbic acid, some quantity of bioflavinoid.

The complete agreement of the curves of decrease of η_{rel} of the substrate under the influence of hyaluronidase alone and hyaluronidase with additions (Fig. 4) shows at once that bioflavinoids cannot "develop" an action of ascorbic acid which is absent.

Somewhat different results were obtained in a study of the effect on hyaluronidase of a preparation of the catechins of tea leaves, which have vitamin P activity [24]. It was shown that tea leaf catechins have an antihyaluronidase activity the degree of which depends directly on duration of contact with hyaluronidase. Therefore we directed out attention to the dynamics of the decrease of viscosity with slowing at the beginning and marked hastening after 5-15 min from the start of the reaction (Fig. 5). Using the catechins with ascorbic acid caused a decrease in inhibitory action of the catechins,

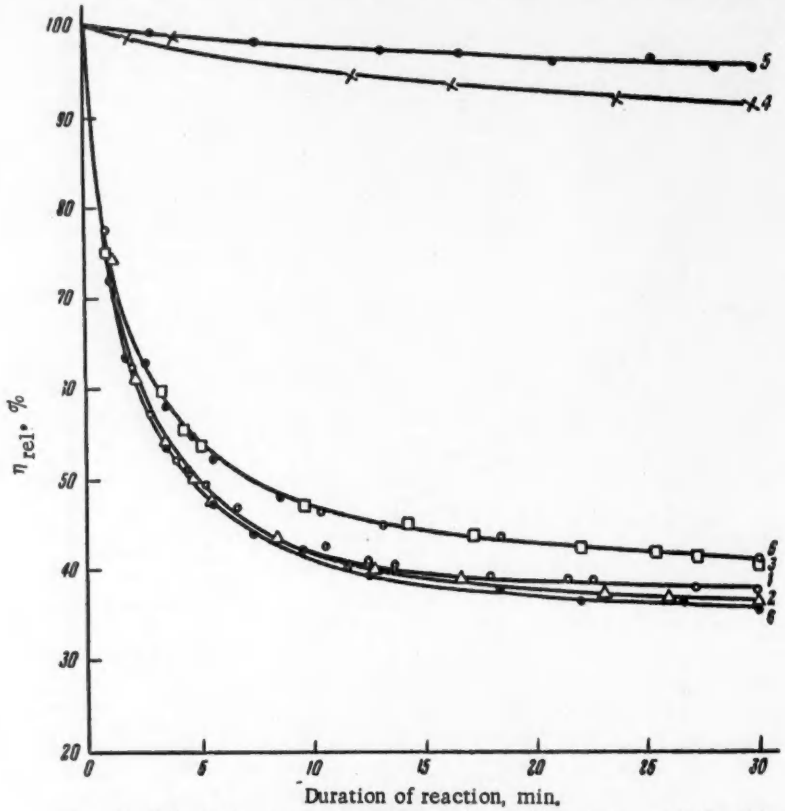

Fig. 3. Effect of enzymatic oxidation of ascorbic acid on activity of hyaluronidase. Percent oxidation of ascorbic acid in the sample: 1) 20%, 2) 50%; 3) 100%; 4) 50% (without hyaluronidase). Samples without ascorbic acid: 5) Inactivated hyaluronidase; 6) hyaluronidase.

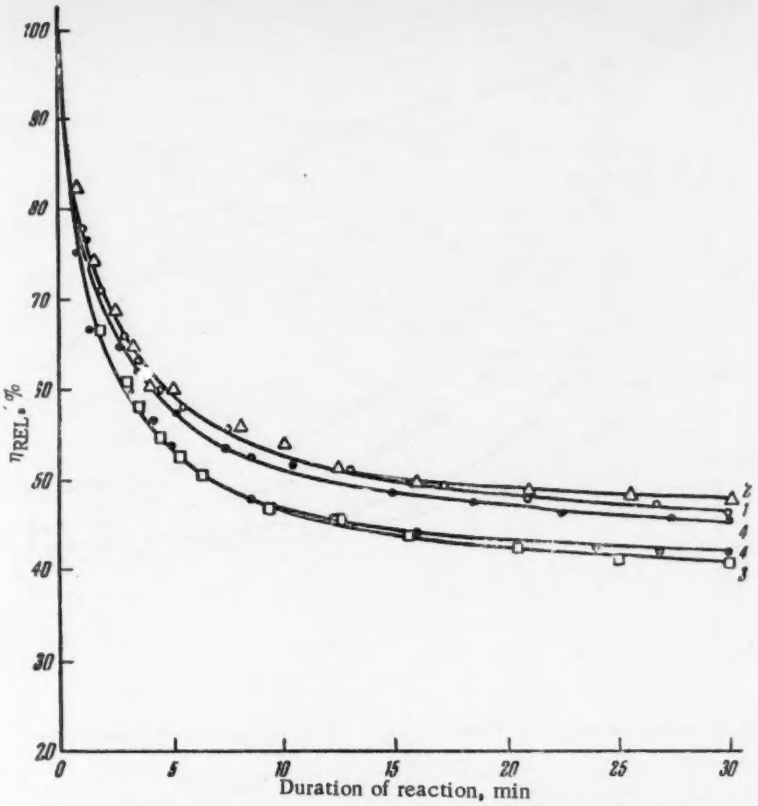

Fig. 4. Effect of bioflavinoids and ascorbic acid on the activity of hyaluronidase. Preliminary incubation 90 min: 1) Ascorbic acid + rutin; 2) ascorbic acid + esculin; 3) ascorbic acid + hesperidin methylchalcone; 4) without additions.

which suggests rather a protective action of ascorbic acid on hyaluronidase. A preliminary keeping for an hour of the bioflavinoids or their mixture with ascorbic acid and substrate did not inhibit enzymatic depolymerization of the latter. Thus, evidently, under our experimental conditions there was no antihyaluronidase action of ascorbic acid and the bioflavinoids. The inhibition of hyaluronidase with time by the catechins of tea leaves is probably due to a nonspecific effect and depends on the presence of admixtures.

Considering the data on nonhomogeneity of the hyaluronidase, we suggest that the negative results obtained in our experiments may depend on the choice of method for determining hyaluronidase activity and that the two methods, viscosimetric and turbidimetric, actually determine two different activities. In order to exclude this possibility, we decided to repeat the investigation of Beiler and Martin, who used the turbidimetric method in their work for determining the hyaluronidase activity. Therefore we ran a series of experiments by the plan described in the methodological part of this study. At the same time we ran determinations with hyaluronic acid and hyaluronidase preparations from the firm of L. Light and Co., which excluded the possibility of the dependence of the results of the enzyme reaction on the properties and quality of the enzyme or substrate.

We showed that both the commercial enzyme preparation and the hyaluronidase which we prepared were insensitive to the same extent to the addition of ascorbic acid had identical activities (Table 2, I). In Table 2 we give data from the turbidimetric method of determination, where the activity of hyaluronidase is expressed by the direct reading of the scale of the nephelometer (inversely proportional to the absolute size of the value of activity).

The same values for the nephelometer readings in measuring the turbidity of different samples indicates the presence of the same amounts of undecomposed hyaluronic acid and so of equal hyaluronidase activity.

In Table 2, II, we give average data for for some determinations of the activity of hyaluronidase carried out on preparations of hyaluronic acid from the firm of Light and Co. in the absence of NaCl in the enzyme mixture, which show that ascorbic acid has no protective action on the substrate. Analogous experiments carried out on our own preparation of substrate showed that to produce a marked decrease in turbidity under these conditions we had to have a larger amount of enzyme (about 0.4 mg), but this had no effect on the final result of the

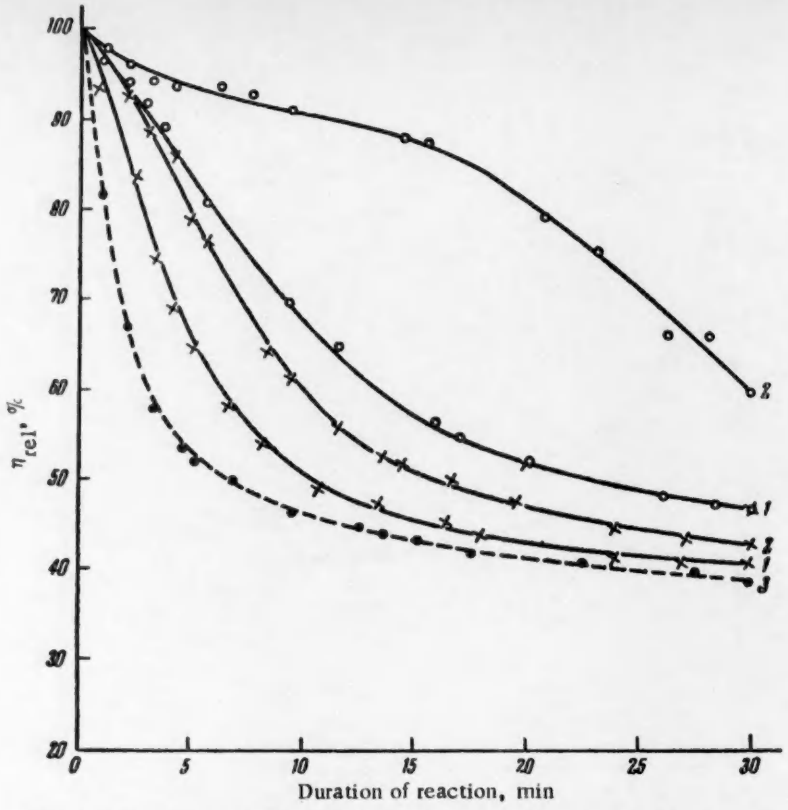

Fig. 5. Effect of incubation of hyaluronidase with tea leaf catechins and ascorbic acid on enzyme activity. Preliminary incubation, min: 1) 60; 2 and 3) 300; circles—catechins; crosses—catechins + ascorbic acid; dotted line—without additions.

TABLE 2.

Effect of Ascorbic Acid on Activity of Hyaluronidase Acetate buffer, pH 6.0, volume of mixture 2.0 ml; hyaluronic Acid 0.8 mg; I, III—our preparation; II—preparation of Light and Co.

Expt.	Hyaluronidase		Final concentration of NaCl, M	Ascorbic acid, mg in sample	Nephelometer reading
	preparation	μg in sample			
I	Our preparation	500	0,09	1,0	0,073
		500	0,09	0	0,064
	Light preparation	500	0,09	1,0	0,050
		500	0,09	0	0,061
II	Our preparation	100	0	0,5	0,364
		100	0	0	0,364
	Without Enzyme	0	0	0,5	0,566
		0	0	0	0,521
III	Our preparation	100	0,09	0,5	0,026
		100	0,09	0	0,028
	Our preparation	25	0,09	0,5	0,367
		25	0,09	0	0,365
	Without enzyme	0	0,09	0,5	0,478
		0	0,09	0	0,437

reaction: the inhibiting effect of the ascorbic acid on the hyaluronidase was absent. Thus the negative result of our investigation does not depend on the presence in one of the preparations of hyaluronic acid of contaminants which inhibit the action of hyaluronidase.

The results of the reaction are not affected by use of different preparations of hyaluronic acid, addition of 0.09% NaCl, increase in ascorbic acid concentration, or decrease in amount of enzyme. Comparisons of our data led rather to the opinion of an activating action

of ascorbic acid. However, this seeming increase in hyaluronidase activity is explained by the higher control in the presence of ascorbic acid in the substrate solution and by its possible share in the formation of supplementary turbidity.

DISCUSSION OF RESULTS

The viscosimetric method for determining hyaluronidase activity is a sufficiently rapid and promising method which gives reproducible results. In comparison with the turbidimetric method, viscosimetry has a number of advantages, the most important of which is the more direct estimation of the results of enzyme action; this is especially important for showing the activating or inhibiting properties of various substances.

The turbidimetric method in the variant used by Beiler and Martin [3] is a rather imperfect process whose poor reproducibility cannot be due entirely to the properties of the preparations of hyaluronidase and hyaluronic acid used, since merely a heat stabilization of the serum protein removes all the side effects and greatly improves their results. The question arises whether the inhibiting action of ascorbic acid observed by Beiler and Martin depends on its reaction with the unstabilized protein. Naturally the reaction of native hyaluronic acid (control determination) and substrate, partly decomposed by hyaluronidase (experimental determination) with acidified serum protein in the presence of ascorbic acid may be different and may also reflect on the results of the investigation. Also it is very significant that the source of the hyaluronic acid in the work cited was the vitreous body of beef eyes, while in our work we used hyaluronic acid extracted from the umbilical cord of the newborn which contains a definite proportion of chondroitin sulfuric acid. This is especially important to remember in view of the poor agreement of the results of investigations carried out in different laboratories on different preparations of substrate and enzyme, which has been emphasized by many investigators [22].

According to the data of Beiler and Martin, the inhibiting action of rutin in 0.1% concentration is 70% too high a concentration to speak of specificity of reaction, and the effect of ascorbic acid is still higher. Although there is no apparent reason to doubt the validity of the results, the agreement of our data obtained by viscosimetric and turbidimetric methods for determining the activity of hyaluronidase permits us to consider that neither ascorbic acid nor bioflavinoids act as inhibitors of hyaluronidase. The inhibition of hyaluronidase by the catechins of tea leaves is nonspecific and confirms the above opinion since if these substances were inhibitors, they would certainly show it.

All these facts indicate the existance of a complex relationship between hyaluronic and ascorbic acids. On the one hand, ascorbic acid had the properties of a diffusing factor [25] and by its oxidation lowers the viscosity of hyaluronic acid; on the other, we have shown in our work that hyaluronic or chondroitin sulfuric acids apparently protect the vitamin from oxidation by ascorbic oxidase. The explanation of the mechanism of the protective action of ascorbic acid on hyaluronidase when it is acted upon by tea leaf catechins will be which causes inhibition of the enzyme. All these facts do not contradict our basic conclusion of the absence of an inhibiting action of ascorbic acid on hyaluronidase in vitro. However, in experiments on the intact organisms with simultaneous injection of hyaluronidase and ascorbic acid or bioflavinoids, other results may be obtained [5, 6], which indicates the occurrence of side or secondary mechanisms which are difficult or even impossible to produce in an in vitro experiment.

On the basis of the data given the reason for the destruction of permeability with insufficient ascorbic acid and bioflavinoids must evidently be sought in the damage to synthesis and change in the state of the chief intercellular substances of connective tissue and collagen. At the present ime there is no doubt of the participation of ascorbic acid in the synthesis of collagen and the metabolism of a number of mucopolysaccharides which make up the main mass of intercellular substances and share in the formation of collagen fibers. With a deficiency of ascorbic acid in the organism, destruction of all these processes begins. Along with the facts which indicate the absence of synthesis of hyaluronic acid in the granulating tissues of the guinea pig in scurvy there is evidence that with this there is accumulation of unusual sulfur-free polysaccharides identical with hyaluronic acid [10]. Processes of formation of collagen fibers are damaged, tissues are weakened, the permeability of intercellular membranes and capillaries is increased. The process of collagen formation when ascorbic acid is supplied is accompanied by rebuilding of mucopolysaccharides with the introduction of sulfate into their molecules [26] and transformation of precollagen into collagen as shown by an increase in content of hydroxyproline along with proline and glycine [10, 27]. Here the introduction of sulfate precedes its activation with the participation of ATP and Mg ions [28, 29] which is hastened several fold by the presence of glutamine. Bioflavinoids, especially the catechins of tea leaves, permit the accumulation of ascorbic acid in all organs and show a direct action on damage to permeability [24]. The process is very complex and occurs with participation of a number of enzymes and hormone systems .

Finally, everything is not yet clear as to the biosynthesis of collagen and mucopolysaccharides nor is there a final explanation of the degree of participation of ascorbic acid and bioflavinoids in this process but it is possible to say that the increased permeability in ascorbic acid and bioflavinoid deficiency can be ex-

plained by damage to the synthesis of the chief elements of connective tissue, destruction of fibrillogenesis which is accompanied by weakening of structure formation, and accumulation of hyaluronic and sulfur-free chondroitin sulfuric acid which make for greater sensitivity to the action of various depolymerizing agents. In such an approach to the problem of damaged permeability the protective effect of hyaluronic acid on ascorbic acid assumes an important physiological significance. This does not exclude the possibility of an inhibitory action of ascorbic acid and the bioflavinoids on hyaluronidase in the animal organism, but only indicates that it is not direct and does not appear in experiments on isolated systems.

SUMMARY

Ascorbic acid and bioflavinoids in themselves do not have the properties of hyaluronidase inhibitors, as is shown by experiments in vitro with purified preparations of substrate and enzyme. The inhibition of hyaluronidase by tea leaf catechins is apparently nonspecific and cannot be connected with their vitamin P properties.

These data do not exclude theinhibiting action of ascorbic acid and bioflavinoids on hyaluronidase in the animal organism from secondary or side reactions.

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THE SIGNIFICANCE OF ASCORBIC ACID IN THE FORMATION OF DEOXYPENTOSE COMPOUNDS UNDER THE INFLUENCE OF EXTRACTS OF ANIMAL TISSUES

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It was shown in previous investigations that ascorbic acid (AA) is a factor which stimulates formation of DNA in cells of the animal organism [1]. It was suggested that AA is a substrate for formation of deoxyribose [2]. A number of indirect reports favor this view. These include the substrate amount of AA in the tissues, the share of AA in the pentose cycle [3], the formation from AA of small amounts of the carbohydrates, L-xylose and glucose [14]. It becomes of interest to explain whether in the tissues there is actual transformation of AA into deoxyribose.

In the present work we have tried to explain whether among the transformation products of AA there are any which give a specific color reaction for deoxyribose. As such a color reaction we used the reaction with cysteine-sulfuric acid suggested by Dische, in the Stumpf modification, and also the reaction with diphenylamine according to Dische [5].

Starting from the fact that the process of formation of DNA can be found in tissues with destroyed cell structures [1,2] we have tried to explain whether the transformation of AA to deoxyribose, or to any substance similar to it, can occur with the aid of cell-free extracts.

METHODS

Determination of deoxyribose was carried out by the reaction with cysteine and sulfuric acid in the Stumpf modification, and also with the aid of the Dische reaction with diphenylamine [5]. The values of the extinction, depending on wave length, and also their maximum were determined with a K-56 spectrophotometer (Prague).

The cell-free extract was prepared by shaking ground tissues of intestinal mucosa and liver of guinea pigs with 1% NaCl. After shaking, the extract was rapidly separated from the tissue by centrifuging at 4000 rpm.

RESULTS AND DISCUSSION

The data which we have obtained (Table) show that on incubation of a solution which contains 100 mg AA with the cell-free extract of intestinal mucosa or liver in the presence of toluene there is formed a substance which gives a clear, sometimes intense, reaction for deoxyribose with cysteine-sulfuric acid. In the spectrophotometric study, the peak of the reaction agrees with the maximum absorption of the pigment formed in the same reaction with DNA in parallel experiments, that is, it is found at 490 m μ (Fig.). Study of the usual product of AA oxidation, dehydroascorbic acid (DAA) and also the products formed by heating DAA in a neutral medium for 30 min at 100° showed that they give no reaction at all with cysteine-sulfuric acid. The products of deeper oxidation gave a reaction

TABLE

Effect of Extracts of Intestinal Mucosa on Transformation of AA, RNA Ribose, and F-1, 6-P into "Deoxyribose". Increase of "deoxyribose" compared to control, %; control is sample of the same composition without incubating; incubation 24 hours at 37°; total volume of sample −4 ml

Composition of sample	Number of experiments	Average data
2 ml extract + H_2O	9	40
2 ml extract + 100 mg AA	9	73.9
2 ml extract + 10 mg RNA	9	264
2 ml extract + 1.9 micromole F-1,6-P	9	82

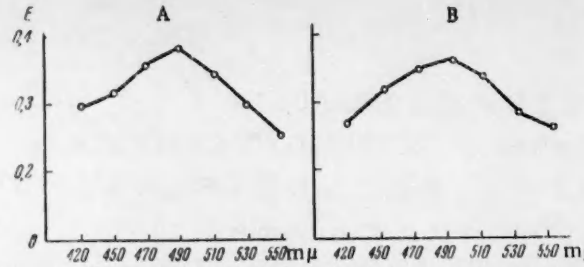

Figure. Absorption spectra of products formed after incubation of mucosa extracts with ascorbic acid (A) 100 mg, and DNA (B) in the reaction with cysteine and sulfuric acid.

with a maximum absorption by the color which lies in the region of 400 mμ. In other words, the generally known oxidation products of AA: DAA, diketogulonic acid, threonic acid, and oxalic acid, are not the substances which are formed in our experiments and which give the reaction for deoxyribose with cysteine-sulfuric acid. We should note the specificity of this reaction, as mentioned by Dische.

The resulting products do not give the reaction with diphenylamine characteristic for deoxyribose reaction, or, in any case, a very slight one. We should mention that this reaction is masked by AA, an excess of which is always contained in the reaction mixture and which gives a brown color with diphenylamine. How can we explain the fact that in the transformations of AA under the influence of tissue extracts products are formed which give the more specific reaction for deoxyribose with cysteine-sulfuric acid and do not give the less specific, though more sensitive reaction with diphenylamine? Here we should always consider the possibility of formation of compounds structurally close to deoxyribose, which can also give the reaction with cysteine-sulfuric acid. For example, Dische shows that arabinal gives such a reaction [5]. Besides this, arabinal can be transformed into deoxyribose [6]. It may be that we are here dealing with this or some other similar compound. On the other hand, it is known that pyrimidine deoxyribosides (particularly thymidine) do not give the reaction for deoxyribose with diphenylamine. Besides this, with cysteine-sulfuric acid thymidine gives a clear positive reaction characteristic for deoxyribose [5]. Cannot these results be explained by the fact that the process of transformation of AA occurs first by the formation of a compound between AA and thymine, which then reacts further to form thymidine? This view is apparently supported by experiments in which the method was used of breaking the bonds between the pyrimidine bases and deoxyribose with the aid of bromination [7].

After such treatment of the reaction mixture obtained by incubation of the tissue extracts with AA, we find the appearance of the reaction with diphenylamine for deoxyribose. The increase of deoxyribose over the control is 63.9% (average of 7 experiments).

It is interesting that the formation of products which give the positive reaction for deoxyribose with cysteine and sulfuric acid occurs when the tissue extracts are incubated not only with AA, but also with RNA and fructose-1,6-diphosphate (F-1,6-P). The products formed as a result of transformations of the latter two substances also fail to give positive reactions with diphenylamine, but give their reaction with cysteine-sulfuric acid. The maximum absorption of the color of this reaction in the spectrophotometric study also agrees with the maximum absorption in the reaction with DNA. Thus, the identity, or at least the close similarity of the products of transformation of such different compounds as AA, RNA ribose, and F-1,6-P apparently favors the formation in all three cases of deoxyribose or similar and genetically related compounds, since the latter two compounds (ribose and F-1,6-P) certainly can serve as precursors of deoxyribose in the cells of animal organisms. We should mention that quantitatively, as the table shows, the intensity of transformation of RNA ribose into the compound which we have conditionally called "deoxyribose" considerably surpasses the intensity of transformation of AA and F-1,6-P into the same compound. This fact, we think, indicates that its transformation is completed with the participation of other components of the nucleic acid besides the carbohydrates, especially the nitrogenous bases which are contained in RNA and are absent in AA and F-1,6-P.

It is also important to mention some characteristics of the reaction of formation of "deoxyribose" when tissue extracts are in incubated with AA. A study of the effect of mucosa extracts on the formation of "deoxyribose" in the presence of different concentrations of AA showed the following:

Extracts with Addition of AA Amounts

Inc. in "deoxyribose", %	Extracts with addition of AA in amounts
4.1 (8)	10 mg
17.8 (8)	50 mg
73.9 (9)	100 mg
132 (4)	200 mg
199 (4)	400 mg

Note: Figures in parentheses are number of experiments. For conditions of incubation, see Table.

Thus, the formation of "deoxyribose" occurs only in the presence of a considerable amount of AA, not less than 100 mg in 4 ml of reaction mixture; in the presence of 10 mg, formation does not occur, and, in the presence of 50 mg, it is very slight. Further increase in concentration of AA leads to increase in intensity of the reaction.

During the reaction AA disappears, and after 24-hour incubation not more than 50% of the original amount can be found. Simultaneously there appears a considerable amount of diketogulonic and dehydroascorbid acid (determined by the method of Roe and Kuether). However, as was noted above, these compounds do not give the reaction with cysteine and sulfuric acid which is characteristic for "deoxyribose."

We tried to determine whether AA could be considered as an activator of other reactions in which the substrate for the formation of "deoxyribose" was RNA or F-1, 6-P. For this purpose incubation of the tissue extract was carried out in the presence of AA + RNA and AA + F-1,6-P. As the control we used the extract with addition of water. The results were as follows:

To 2ml of extract added	Inc. in "deoxyribose", %
AA, 19 mg	6.9 (5)
AA, 100 mg	103 (6)
RNA, 10 mg	322 (6)
F-1,6-P, 1.9 μ moles	119 (7)
To 2ml extract added	**Inc. in "deoxybibose", %**
AA + RNA, 10 mg	251 (5)
AA 10mg + F-1, 6-P 1.9 μ moles	71 (2)
AA 100mg + F-1,6-P, 1.9 μ moles	215 (5)

As these data show, under the influence of AA not only is there no activation of the process of formation of "deoxyribose" from RNA or F-1,6-P, but, on the contrary, rather these reactions inhibit each other. Thus, the AA concentration required in the reaction mixture (corresponding to the concentration of a substrate and not an activator), the dependence of the rate of reaction on AA concentration, the disappearance of considerable amounts of AA from the reaction mixture during the reaction, and also the impossibility of detecting an activation by AA of any other reaction of formation of "deoxyribose" force us to conclude that in the reaction studied AA apparently plays the role of a substrate and not an activator.

We must keep in mind that in the tissues we certainly will find several paths of formtion of deoxyribose and DNA [8], each of which will be specific and probably will have definite biological value.

SUMMARY

When cell-free tissue extracts of intestinal mucosa and liver of guinea pigs are incubated at 37° in the presence of ascorbic acid, ribonucleic acid, and fructose diphosphate they form compounds which give a positive reaction for deoxyribose with cysteine-sulfuric acid. This compound apparently contains deoxyribose or another structurally closely related substance.

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A STUDY OF THE AMIDASE ACTIVITY OF BACTERIA AND THE EFFECT ON IT OF SOME CHEMOTHERAPEUTIC SUBSTANCES

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Among the amidases of microbial origin there have been described enzyme systems of micoorganisms which catalyze the hydrolysis of glutamine, asparagine [1-9] and other amides of amino acids [9-11]. Less studied have been the amidases which hydrolyze acetamide, nicotinic acid amide, and other amides [9,12,13]. We have set ourselves the problem of studying in some bacteria the presence and properties of enzymes which deaminate acetamide and nicotinic acid amide, and of studying the effect on these enzymes of antimicrobial preparations.

As objects of microbial study we used blue green pus bacteria as a resting suspension, and acid resistant saprophytes B5 (Mycobacterium n. sp.) as an acetone preparation and as a resting suspension. The enzyme activity was determined by the evolution of ammonia. The bacterial suspensions and acetone preparation of the B5 bacteria were prepared by the methods described earlier [14, 15].

METHODS

Determination of amidase activity of the microbes. In the central chamber of a Conway glass vessel was placed 1.6-2 ml of 0.1 N H_2SO_4. In the outer chamber of the control vessel was placed 1 ml of a 50 billion suspension of blue green pus bacteria, 2 ml of 1/15 M phosphate buffer, and 1.5 ml of a solution, or a weight, of the substance to be studied in an alcoholic solution of phenol as an antiseptic. In the outer chamber of the vessel which contained the object under investigation was placed 1 ml of microbe suspension, 2 ml of phosphate buffer, 1 ml of 0.2 N solution of acetamide, and 2 drops of 50% alcoholic solution of phenol. After incubation for 24 hours at 37°, 4 ml of saturated potash solution was placed in the outer chamber, and the vessel was again kept at 37° for 4 hours; then the contents of the central chamber were titrated with 0.1 N NaOH with methyl red as indicator.

The measure of amidase activity was the amount of acid required to neutralize the ammonia formed in the enzymatic process. If nicotinic acid amide was used as the substrate, we used 0.025 N H_2SO_4 and correspondingly, 0.025 N NaOH. The activity of the suspension of bacteria B5 was determined in an analogous way. The amount of bacteria in suspension in this case was 200-300 mg/ml*. We used 0.05 N H_2SO_4 for acetamide and 0.1 N for nicotinic acid amide. In work with the acetone preparation of B5 bacteria we used 160 mg of the preparation in the sample. H_2SO_4 was used in 0.1 and 0.05 N solution.

In all cases we also determined the activity of the boiled bacterial suspension (or weight of acetone powder from the bacteria). These did not have enzyme activity.

Study of the effect of bacteriostatic substances on amidase activity. In the outer chamber of the Conway vessel we placed 1 ml of bacterial suspension., 0.5 of 1/15 M phosphate buffer, and 1.5 ml of solution, or weight, of the substance to be studied (the solution was prepared in the buffer); the mixture was carefully stirred and the vessel was closed and kept for 2 hours in the thermostat at 37°. For calculation of the concentration of the substance under investigation we considered the dilution resulting from addition of the bacterial preparation. We also placed in the thermostat for 2 hours a vessel which contained instead of the studied substance a buffer solution (we determined the initial amidase activity of the microbe suspension in this vessel). After the preliminary two hour incubation we added to the outer chamber of the Conway vessel 1 ml of 0.2 N solution of acetamide or nicotinic acid amide, and to the control vessel 1 ml of distilled water, and to the central chamber, 1.6 to 2.0 ml of H_2SO_4 of the corresponding concentration. The amidase activity was determined by the above method.

RESULTS OF THE INVESTIGATION

Properties of Amidase

The suspension of blue-green pus bacteria and the suspension and acetone preparation of acid resistant saprophyte B5 had marked amidase activity toward acetamide and nicotinic acid amide. The activity of the bacterial amidase changed with the pH of the medium. The maximum activity of the studied aminade occurred at pH 8-9; in an acid medium amidase activity was greatly decreased (Fig. 1).

* The density of the suspension was determined by turbidity, according to the BCG standards.

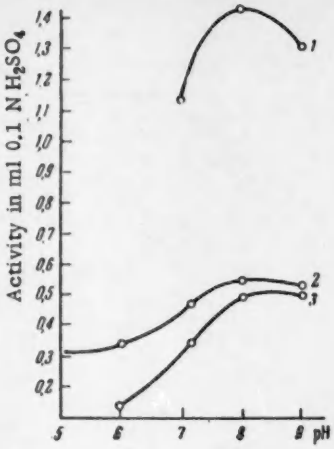

Fig. 1. Effect of pH of the medium on amidase activity. 1) Suspension of pyocyanic bacteria + acetamide; 2) acetone preparation of B5 + acetamide; 3) acetone preparation of B5 + nicotinamide.

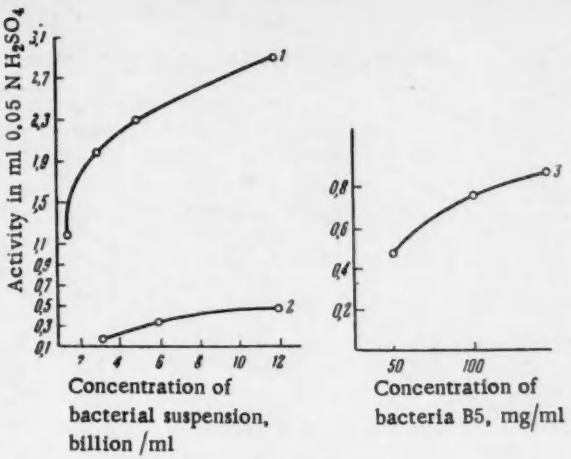

Fig. 2. Effect of concentration of enzyme material on amidase activity. 1) Suspension of pyocyanic bacteria + + acetamide; 2) suspension of pyocyanic bacterial+ nicotinamide; 3) suspension of bacteria B5 + nicotinamide; on the ordinate – activity in ml 0,1 N H_2SO_4.

TABLE 1

Dependence of Amidase Activity on Concentration of Substrate. Acetone Preparation of Bacteria B5 and Acetamide

Amount of substrate in sample, moles	Activity in ml 0,1 N H_2SO_4	Deamination, %
$5 \cdot 10^{-5}$	0,32	64
$1 \cdot 10^{-4}$	0,5	50
$15 \cdot 10^{-5}$	0,73	48,6
$2 \cdot 10^{-4}$	0,82	41
$3 \cdot 10^{-4}$	0,88	29

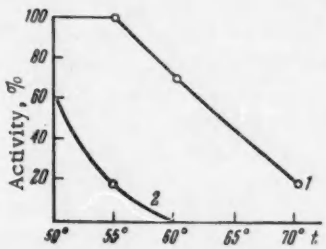

Fig. 3. Effect of temperature on amidase activity of acetone preparation of bacteria B5. Exposure, 5 min. 1) Substrate – acetamide ($1 \cdot 10^{-4}$ M); 2) substrate – nicotinamide ($1 \cdot 10^{-4}$ M).

The amidase activity of the bacterial suspensions changed with concentration of enzyme material (Fig. 2). Increase in concentration of the substrate in the experiments caused increase (to a definite limit) in the amount of reacting amide, but at the same time the amount of amide which entered reaction decreased (Table 1).

Study of the kinetics of the reaction showed that, depending on the nature of the enzyme and the concentration of the substrate, the enzymatic process reached equilibrium between 20-24 and 28 hours, and in some cases, after 32-40 hours.

Suspensions obtained from the acetone preparation of bacteria B5 after heating on the water bath very easily lost amidase activity with respect to nicotinic acid amide. Heating the suspensions for five min at 50° decreased the enzyme activity by 40%, and at 55°, by 80%; five minute heating at 60° inactivated the enzyme completely. The enzyme which hydrolyzes acetamide was less sensitive to temperature; heating a weight of the acetone preparation of bacteria B5 for 5 min at 50-55° did not affect the activity of the enzyme; heating for the same period at 60° caused 25-30% inactivation; at 70°, 80-85% (Fig. 3). When the bacterial suspensions were kept for long periods there was also rapid inactivation of the enzyme which caused hydrolysis of nicotinamide. These results show that the deamidation of acetamide and nicotinamide are catalyzed by different enzymes.

The age of the B5 bacterial culture evidiently has no important effect on the activity of the amidase. Thus, the acetone preparation of the B5 bacteria taken from three, four, five, six, and seven day old cultures had the same amidase activity.

TABLE 2

Inhibition of Amidase Activity by 3-Amino-4-Hydroxyphenylarsine Oxide (in %)

Concentration of preparation	Weight of acetone preparation of bacteria B5		Suspension of bacteria B5		Suspension of pyocyanic bacteria	
	I	II	I	II	I	II
1 : 1000 — 1 : 2000	25—35	35—45	10—25	25—30	90—100	70—75
1 : 4000	20—25	20—25	0	24	80	—
1 : 8000	0	18	—	10	—	—
1 : 16 000	—	0	—	0	—	—
1 : 20 000					60	30—40
1 : 200 000					27	10—20
1 : 2 000 000					17	0
1 : 20 000 000					0	

Study of the Effect of Chemotherapeutic substances on the Amidase Activity of Bacteria

We studied the effect on the amidase activity of the B5 bacteria and the pyocyanic bacteria of the following chemotherapeutic substances: white streptocide, sulfidine, sulfazole, synthomycin, aureomycin, penicillin p-amino-salicylic acid, tubaside, phthivazide, tibone, cutisone, quinine, acrichine, naganine, surmin, tripaflavine, and 3-amino-4-hydroxyphenylarsine oxide. We also studied the effect on amidase activity of the pyocyanic bacteria of saluzid and metazid. The enzyme which hydrolyzed acetamide showed some sensitivity only to 3-amino-4-hydroxyphenylarsine oxide (Table 2); the other preparations showed no effect on the activity of this enzyme.

Somewhat greater sensitivity to the preparations studied was shown by nicotinamidase. Sulfidine and sulfazole in concentrations of 1: 1000 slightly (10-20%) suppressed the nicotinamidase activity of bacteria B5 (as the acetone preparation and also as the suspension). Aureomycin in high concentrations (1: 1000 to 1: 2000) lowered slightly (10-20%) the nicotinamidase activity of the suspension of bacteria B5. Tripaflavine at concentrations of 1: 1000 to 1: 2000 lowered slightly (10-20%) the activity of nicotinamidase of pyocyanic bacteria and bacteria B5. A high concentration of 3-amino-4-hydroxyphenylarsine oxide completely or partially activated the nicotinamidase of pyocyanic bacteria and bacteria B5 (Table 2). The nicotinamidase of pyocyanic bacteria was partly inhibited by tibone and cutisone (Table 3). The same preparations did not act on the amidase activity of bacteria B5.

TABLE 3

Inhibition (in %) of Amidase Activity of Pyocyanic Bacteria by Antimicrobial Preparations. Substrate — Nicotinamide

Concentration of preparation	Tibone	Cutisone
1 : 2 000	80	70
1 : 20 000	80	62
1 : 2 000 000	67	44
1 : 20 000 000	40	19
1 : 40 000 000	20	13
1 : 80 000 000	Does not inhibit	7

Among the compounds studied, only tibone in the minimum bacteriostatic concentrations showed some effect on the nicotinamidase of the pyocyanic bacteria; the other preparations at these concentrations did not act on the amidase activity of bacteria. Evidently, the enzymes which hydrolyze acetamide and nicotinamide are not sensitive, or are little sensitive to the preparations studied and the antimicrobial action of these preparations is not connected with the inhibition of the enzyme systems which hydrolyze acetamide and nicotinamide.

SUMMARY

Resting suspensions of pyocyanic bacteria, acetone preparations from mycobacteria B5, and resting suspensions of mycobacteria B5 have amidase activity toward acetamide and nicotinic acid amide.

The studied amidases have maximum activity at a pH of the medium of 8-9. With increased concentration of enzyme and substrate to definite limits, the rate of deamidation increases. Depending on the nature and concentration of the substrate, the enzymatic processes reach equilibrium after 20-24 and 28 hours, and in some cases, after 32-40 hours.

The amidase activity of bacteria B5 does not depend on age of the cultures. The amidases studied in this work are thermolabile. Nicotinamide amidase is more sensitive to temperature than is acetamide amidase. Acetamide amidase is also more stable when kept.

These bacterial amidases are little sensitive to the effects of chemotherapeutic substances. The antimicrobial action of the chemotherapeutic compounds studied cannot be related to their effects on the amidase activity of the microorganisms.

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THE CATECHINS AND FLAVONOIDS IN CERTAIN TYPES OF TEA OF THE CHINESE PEOPLE'S REPUBLIC

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The interest in Chinese tea studies has shown a notable increase since the establishment of the Chinese People's Republic. As contrasted with other counteries, there are produced not only green and black Bohea tea, but also a number of other types.

It is sufficient to say that export tea alone accounts for over 60 brands, while those for domestic consumption consist of more than 100 types. The kinds of tea produced in the Chinese People's Republic differ from each other in appearance as well as in their organoleptic qualities, depending on the kind of raw material used and the character of processing. The issuing material for certain types consists only of growing buds (Baicha Inchzhen), for others of the growing buds with the first leave (Luntszin), for a third type, of the tender tips with one or two leaves, where the top bud is dead (Ulun), etc. The processing markedly differs also. Thus, the preparation of one type requires a rapid thermal treatment of the raw material (green tea), while other types require thermal treatment of the wilted leaf (Inchzhen) or a fermentation process (black tea), etc.

During his stay in the Chinese People's Republic, K.M. Dzhemuchadze had an opportunity to become acquainted with the preparation of various types of tea, as well as to collect samples.

The study of the various types of tea showed that they all differ in chemical composition, and should therefore differ also in dietetic and physiological properties.

In our paper are presented results obtained from the study of catechins and flavonoids of several of the most popular types of tea of the Chinese People's Republic.

METHODS

The catechins were determined separately by the quantitative method of paper chromatography [1]. The flavonoids were determined together by the boric acid color reaction, in the presence of citric acid; the standard curve was figured out on the basis of isoquercitrin [2, 3]. Also, the qualitative composition of flavonoids was investigated by dimeric paper chromatography (using Leningrad rapid paper) in the following solvent systems: a monoplasic mixture of n-butanol – acetic acid – water (40 : 12 : 29) and a 2% acqueous solution of acetic acid. After air drying, the chromatograms were studied under ultraviolet light, and the dark spots of flavonoglycosides and the yellow spots of the free aglycones were marked. Subsequently, the chromatograms were sprayed with a 1% alcoholic solution of $AlCl_3$, and after drying, again surveyed under ultraviolet light, where the glycosides of the flavonoids came out as yellow spots, while the aglycones acquired a greenish fluroescence.

RESULTS AND DISCUSSION

As a result of our investigation, it was established that Chinese teas differ markedly not only as to the total catechin content and their individual ratios, but also in the total amount and composition of flavonoids. It turned out that the green Bohea teas of Chinese origin contain quite a substantial amount of catechins, especially their gallic esters, as well as of flavonoids (Table 1).

This is explained by the fact that in the processing of green Bohea tea, the raw material is subjected to a rapid "roasting", i.e., to the effect of high temperature (150-200°C), during which there takes place a complete inactivation of the enzymes, permitting the catechins and flavonoids of the tea leaf to remain practically unchanged. This is why the finished product of the green Bohea tea retains the color of the leaf, and the infuse is of green color with a slight yellowish shade, and is quite astringent to the taste. Most of the inhabitants of the Chinese People's Republic drink this very tea, which is an effective thirst quencher and stimulant. Evidently, the specific dietetic properties of green Bohea tea are due to its high content of catechins and flavonoids.

The Luntszin tea, which is widely used in the Chinese People's Republic, can also be considered as a green type. Its method of preparation differs from that of the green Bohea tea in that the Luntszin raw material is kept for 24 hours in a cool place, followed by a "roasting" at a temperature of 75-100°C. The data of Table 2 show that catechins and flavonoids also should in this tea.

One can readily see from a comparison of the data of Tables 1 and 2 that the Luntszin tea lags behind the green Bohea tea in catechin and flavonoid contents. This is explained by the fact that during the 24 hours of storage of the raw material the said substances are undergoing changes, which, while being small, are nevertheless sufficient to convey to the infuse of the finished product a specific color, a pleasant taste, and a delicate aroma. The Luntszin tea has been highly valued by the Chinese people since early times. But, quite aside from its high

TABLE 1

Catechins and Flavonoids in the Chinese Green Bohea Tea

Catechins	mg/g of dry wt.	In % of total catechins
L-epigallocatechin	7.57	7.44
DL-gallocatechin	5.26	5.17
L-epicatechin and DL-catechin	3.65	3.58
L-epigallocatechingallate	67.01	65.83
L-epicatechingallate	17.11	16.79
Total catechins	101.79	100.0
Flavonoids	11.57	–

TABLE 2

Catechins and Flavonoids in Luntszin Tea

Catechins	Mg/g of dry wt.	In % of total catechins
L-epigalloeatechin	5.89	7.34
DL-gallocatechin	2.20	2.74
L-epicatechir and DL-catechir	5.98	4.01
L-epigallocatechingallate	51.21	63.80
L-epicatechingallate	14.91	18.60
Total catechins	80.19	100.0
Flavonoids	10.44	

organoleptic qualities, the Luntszin tea is of great interest as a source of Vitamin P, due to the high catechin and flavonoid contents.

Also of interest is the "Baicha" tea (white tea), which is widely used in the Chinese People's Republic. The technology of its production is quite simple: The raw material undergoes a prolonged wilting process (48 hr), followed by drying at a temperature of 80-90° C for a period of 20-40 min. The finished product possesses a specific highly delicate flower aroma, a pleasant taste, and its infuse is of a very pretty color. While in the process of Baicha tea preparation the raw material is not subjected to rolling and fermentation, the prolonged wilting stage nevertheless causes substantial changes in the composition of catechins and flavonoids (Table 3).

It can be seen from Table 3 that this variety of tea stands far below the green Bohea tea and the Luntszin tea in catechins, and especially in flavonoids. The comparatively high content of gallic esters and especially of L-epigallocatechingallate is characteristic for the green Baicha tea. This is in good accord with the data [4] showing a high content of L-epigallocatechingallate in the growing bud of the tea plant.

It is hard to say at this time whether such a peculiarity in the composition of catechins affects the dietetic properties of tea, especially its P-vitamin potency. This interesting problem would require a special investigation. There is no question that the Baicha tea exerts a very favorable effect on the human organism, and for this reason is very popular among domestic and foreign consumers. Other types of Baicha tea, which are prepared from different kinds of raw material, differ as to their catechin and flavonoid contents. Thus, the Baimutan type, as compared with the Inchzhen, is much richer in flavonoids, amounting to 13.40 mg/g of dry weight.

A special place among the various types of tea of the Chinese People's Republic is occupied by the Ulan (Balch Dragon). It is a type which lies between the green and black tea, since, during its preparation, the raw material undergoes a partial fermentation. The raw material is subjected to a natural or an artificial wilting process, followed by a light rolling, fermentation, and drying. The light rolling causes only the edges of the leaf to be smashed. These portions of the tea leaf undergo fermentation, and as a result there are produced dark colored sections around the central portion of the leaf, which preserves its green color. As a result of oxidative processes during such a partial fermentation, the catechins of the leaf undergo substantial changes.

As can be seen from Table 4, the gallic ester of the catechins, and especially L-epigallocatechingallate, are the ones undergoing the most intensive changes. This is in good accord with our earlier findings [5], that during the initial stages of rolling and fermentation of the tea it is the L-epigallocatechin and L-epigallocatechingallate which undergo the most intensive transformations. In looking more closely at the data of Table 4 one can see that the Ulun tea contains large quantities of simple catechins. This is due to the fact that in the selection of raw material for the Ulun tea preference is given to the tips with a dead central bud rather than a normal tip with a growing bud. The Ulun tea possesses a specific strong aroma, is of very good taste, and its infuse has a reddish tint, reminding one of black tea. Its catechin and flavonoid content indicate that it must possess high dietetic properties.

The black tea types of the Chinese People's Republic amount to several dozen kinds. They all differ as to the issuing raw material and the process used. Thus, for instance, some of them are subjected to a prolonged wilting (20 - 40 min), still others, to an artificial wilting, etc. Differences exist also in respect to methods of rolling, fermentation, and drying. Besides, the character of the black Bohea tea is greatly influenced by the quality of the raw material and the variety of the tea plant. This is the reason why the black Bohea tea types of the Chinese People's Republic differ greatly also in their chemical composition.

Table 5 gives data on the catechin and flavonoid contents of two samples of black Bohea tea, the Yunnan

TABLE 3

Catechins and Flavonoids in Baicha Tea of the Inchzhen Type *

Catechins	Mg/g of dry wt.	In % of total catechins
L-epigallocatechin	3.73	6.77
DL-gallocatechin	1.07	1.94
L-epicatechin and DL-catechin	1.59	2.88
L-epigallocatechingallate	41.99	76.20
L-epicatechingallate	6.66	12.10
Total catechins	55.04	100.00
Flavonoids	4.04	-

* The Inchzhen is prepared exclusively from the tender growing buds, collected from the Tabaicha variety of the tea plants.

TABLE 4

Catechins and Flavonoids in Ulun Tea (Type Shuisen)

Catechins	Mg/g of dry wt.	In % of total catechins
L-epigallocatechin	26.48	30.01
DL-gallocatechin	4.52	5.15
L-epicatechin and DL-catechin	7.47	8.51
L-epigallocatechingallate	36.63	41.70
L-epicatechingallate	12.64	14.14
Total catechins	87.79	100
Flavonoids	13.27	–

and Tsimyn types. The black Bohea tea types differ greatly from the previously described green Bohea tea, the Luntszin, Baicha, and Ulun, in the catechin content. This is explained by the fact that the technology of the black Bohea tea includes a full cycle of rolling and mashing of the leaf tissues. It is known that from the moment when the tissues are mashed, there begins the fermentation of tea and the oxidative transformation of tanning substances and catechins, resulting in the characteristic black Bohea tea color of infuse, taste, and also in part, aroma of the finished product. It was found that the Yunnan tea contained twice as many catechins as the Tsimyn tea. There is also a big difference in the ratio of various catechins, especially in the content of gallic esters. This may presumably be explained by the character of the raw material as well as the peculiarities of processing. It is known that the southern varieties of the tea plant are prevalent in the Yunnan province, while the Tsimyn tea is mainly prepared from Chinese medium-leaf varieties. The large content of L-epicatechingallate, as well as L-epicatechin and DL-catechin, is an indication that in the Yunnan tea process the fermentation period is shorter than in the Tsimyn. We have shown [6] that L-epicatechingallate, L-epicatechin, and DL-catechin disappear most rapidly during the fourth-fifth hours of fermentation, thus, actually, towards the end of this process.

The Yunnan and Tsimyn black Bohea tea types also differ from the other kinds of tea in the flavonid content. An investigation of the composition of flavonids by means of dimeric paper chromatography showed (see Figure) that most of the flavonoid components are present in all of the Chinese tea types studied, but there exist also some differences in the range of these compounds, which are evidently caused by the character of the raw material and the peculiarities of processing. The total sum of flavonol-glycosides discovered in Chinese teas amounts of 11.

We have not yet identified these compounds but it is possible to give an approximate characterization of the flavonoid components by comparing our chromatograms with those of the Indian tea extracts, reported in the papers of Roberts [7, 8]. Thus, to judge from the pattern of flavonoids and adjacent polyphenolic compounds, one may assume that spot 1 corresponds to the miricetin-3-glycoside of Roberts' chromatogram, spot 2 to quercetin-3-glycoside (isoquercitrin), spot 3 to campherol-3-glycoside (astragaline), spot 5 to quercetin-3-rhamnoglycoside (rutin), spot 6 to campherol-3-rhamnoglycoside, spot 8 to quercetin-3-rhamnoglycoside, and spot 9 to campherol-3-rhamnodiglycoside. The flavonoid components 4, 4a, 7, and 10 remains so far unidentified, even approximately.

It can be seen from the dimeric chromatogram of green tea that there are present the flavonic components 1, 2, 3, 5, 6, 8, 9 and traces of compound 4, with 1, 3, 6, and 9 dominating. A similar pattern can be obtained also from Luntszin tea, with components 1, 2, 3, 6, 8, 9, and traces of compound 5 in evidence.

In Baicha tea of the Inchzhen type (Chart B) are present the flavonoids 1, 2, 3, 4, 5, 8, 9, and traces of compound 7, with compound 9 predominating, and with substantial amounts of compounds 4 and 8, and traces of free aglycones (Ag). This sample shows a great variety of polyphenols, which are much more abundant than in the other teas under investigation. The Ulun tea, type Shuisen, contains a substantial range of flavonoids, such as components 1, 2, 3, 4, 4a, 5, 6, 6, 8, 9 and 10, as well as aglycones (Chart C).

The chromatogram of the Yunnan black Bohea tea (Chart D) shows the presence of flavonoids 1, 2, 3, 5, and 6. The absence of components 8 and 9, which were always found by us in a number of samples of various origin, is here typical; on the other hand, the chromatograms of Yunnan tea show a spot of chlorogenic acid or acids, which was absent in other samples. We were

TABLE 5

Catechins and Flavonoids of the Yunnan and Tsimyn Teas

Catechins	Yunnan		Tsimyn	
	mg/g of dry weight	in % of total catechins	mg/g of dry weight	in % of total catechins
L-epigallocatechin	0,65	1,81	1,10	7,08
DL-gallocatechin	1,76	4,89	1,93	12,43
L-epicatechin and DL-catechin	4,50	12,53	2,57	16,55
L-epigallocatechingallate	11,37	31,60	6,97	44,90
L-epicatechingallate	17,64	49,10	2,96	19,07
Total catechins	35,92	—	15,12	—
Flavonoids	9,27	—	9,68	—

able to trace an analogous pattern of flavonoids, as well as chlorogenic acid, only in the tips from tea plants of southern origin (Vietnam). This observation is in good agreement with the findings of Roberts [9] that the presence of rhamnodiglycosides of quercitin and kaempherol (spots 8 and 9, resp., of our charts) is characteristic only for the Chinese variety of the tea plant.

Contrary to the chromatograms of Yunnan tea, those of the black Bohea tea Tsimyn disclose the presence of only compounds 2,3,8,9, and traces of compound 6, while the tips of the same variety display a rich assortment of flavonoids, components 1,2,3,5,6,8,9, and traces of compounds 4 and 7. Thus, there evidently are taking place, during the processing of this type of tea, deep qualitative changes in the composition of flavonoids. It is of interest however, that the individual representative flavonoids disappear as such, without, as could have been expected, the formation of free aglycones.

SUMMARY

The types of tea of the Chinese People's Republic differ from each other in the contents of individual catechins as well as flavonoids. The types of tea which were not subjected to fermentation (green tea) are the richest in catechins anf flavonoids. Next are the types of tea where the raw material is not subjected to fermentation, but where they are nevertheless undergo definite biochemical changes, including the catechins (Luntszin, Baicha), due to the step of a storage and wilting. The types of tea which undergo a full process of fermentation (black tea) are the poorest in catechins and flavonoids. Finally, the Ulun tea occupies a special position due to the peculiarities of the raw material and of partial fermentation. Here, simultaneously with a high general content of catechins and especially of flavonoids, some individual catechins are undergoing substantial changes, especially the L-epigallocatechingallate.

The types of tea, which were studied by us, all differ as to the ratio of the individual catechins, as well as by the qualitative assorment of the flavonol-3-glycosides, which, evidently, is largely responsible for their organoleptic and dietetic peculiarities.

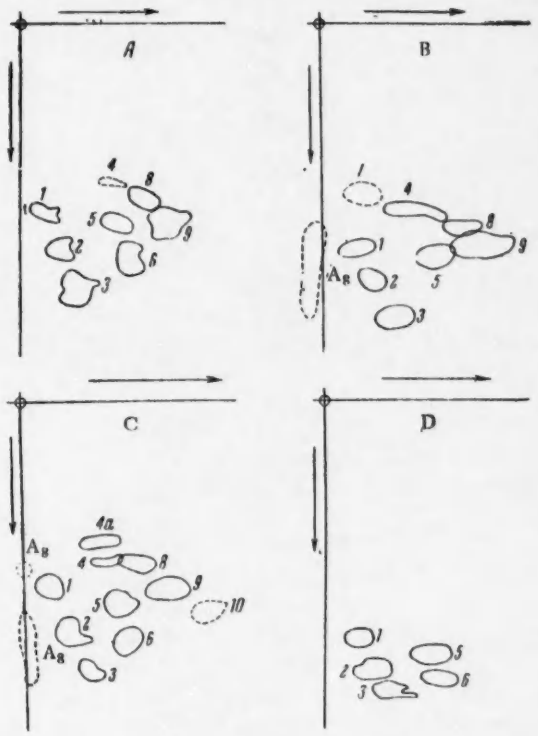

Chart of Dimeric Chromatograms of Various Types of Tea. A) Green Bohea; B) Baicha Inchzhen; C) Ulun Shuisen; D) Black Bohea. Solvents (Going down): n-butanol – acetic acid – water; from left to right, 2% acetic acid. The chromatogram is discussed in the text.

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A STUDY OF THE MECHANISM OF ACTION OF LIVER TRANSGLYCOSYLASE

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The present work is a continuation of the study of liver transglycosylase which has the ability to transfer the glucose residues of dextrin to glucose and other acceptors to form oligo- and polysaccharides of the type of glycogen [1, 2].

This study describes investigations of the mechanism of action of transglycosylase, and particularly includes experiments with maltose which are important in connection with the published work of Stetten [3] showing that the transglycosylase contained in the liver catalyzes the transfer of glucosyl residues of both maltose and dextrin. Since on the basis of our investigations we consider that in the liver there are two transglycosylases, one of which acts on dextrin and the other on maltose, it has become important to test this idea again.

METHODS

The enzyme was isolated from liver tissue as a crystalline preparation [2]. An "alcohol" preparation was also isolated from the liver, which was obtained by homogenizing the liver tissue with 1 volume of alcohol, the precipitate was discarded, the alcoholic extract was treated with four volumes of alcohol, and the precipitate was centrifuged off, dissolved in 0.2 N acetate buffer solution, taken in the amount of 1/10 the volume of the original extract, and the insoluble substance was removed.

The experiments with maltose and trisaccharides were carried out in three variants.

1. The same enzyme preparation was studied separately with chromatographically pure maltose and with trisaccharide with a concentration of each of 10 mg/ml (the same as was used by Stetten); the two incubation mixtures were studied chromatographically and also, in the mixture with maltose, the reduction (before acid hydrolysis) was determined, considering that even a slight increase in this would indicate a positive reaction.

2. The action of highly purified preparations of the enzyme on maltose was studied in parallel with the slightly purified "alcoholic" liver preparation, with determination of the reduction in both mixtures.

3. The enzyme which we isolated was studied in a mixture in which maltose and trisaccharide occurred together, using for this purpose a commercial preparation of maltose which usually contained trisaccharide, or a specially isolated fraction which consisted chiefly of maltose and trisaccharide.

In the experiments with dextrins the activity of the enzyme was determined by the method described earlier [2]. The solvent for chromatographic investigation was a mixture of butanol-ethanol-water (3:2:1) and the developer, aniline phthalate. Magnesium was determined according to Gadient.

For removal of free sugars in the determination of the average unit length of the saccharide chain, the TCA extract of the sample was submitted to the action of a special yeast (Saccharomyces pombe Linder) and the residues of unfermented sugars in the solution were determined by reduction before and after acid hydrolysis, from which we estimated the average chain length of the saccharides.

RESULTS AND DISCUSSION

Experiments with maltose and trisaccharide. The data given in Table 1 show that incubation of three different preparations of transglycosylase with chromatographically pure maltose gives no increase in reduction (Table 1, experiment A, 1-3), even after a prolonged time interval (3 days), while in the mixture with α-dextrin there is a comparatively rapid change in reduction, namely, a decrease (denoted by a minus sign) which shows the active state of the enzyme preparation (the data for one of these experiments is given in experiment B, Table 1).

In parallel investigations of the action of highly purified transglycosylase and the slightly purified "alcoholic" preparation from the liver we observed that the transglycosylase preparation mixed with dextrin caused a definite change in reduction of the sugars in the extract and the precipitate, but did not show any action on the maltose, since the reduction remained unchanged. The "alcoholic" preparation was active with respect to both dextrins and maltose, causing splitting in the latter case and increase in reduction. Thus, these experiments showed that the preparation of highly purified liver transglycosylase contained an enzyme which acted only on dextrins, and in the "alcoholic" liver preparation both enzymes were present, that acting on dextrins and that acting on maltose.

In the study of the incubation mixtures by the paper chromatographic method analogous results were obtained. Thus, in Fig. 1 A we see that in the incubation of transglycosylase with pure maltose its spot remains

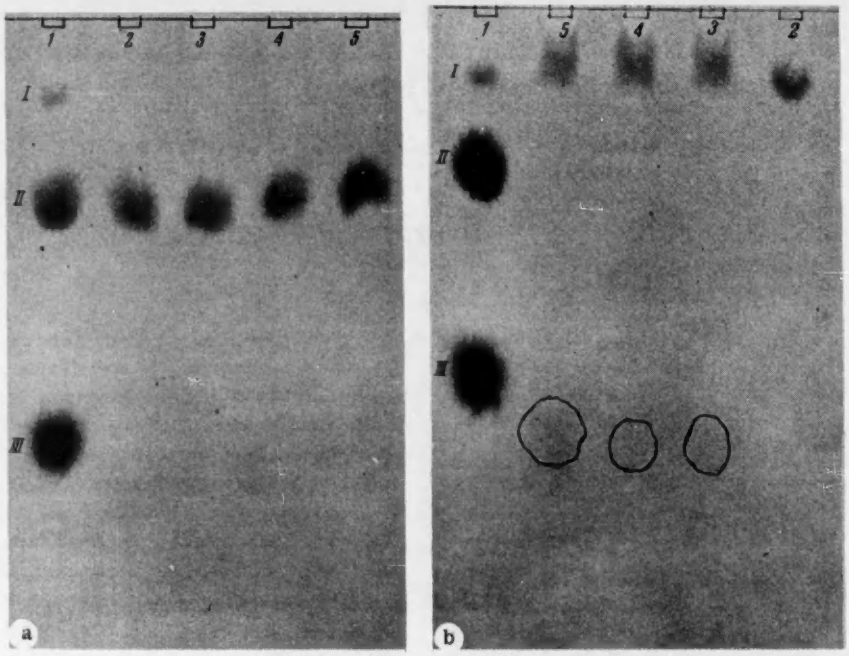

Fig. 1 Chromatographic investigation of the action of transglycosylase on maltose (A) and trisaccharide (B). 1)Marker; 2) reaction mixture before incubation; 3,4,5) the same after incubation after 2 hours, 1and 2 days. I) Trisaccharide; II) maltose; III) glucose.

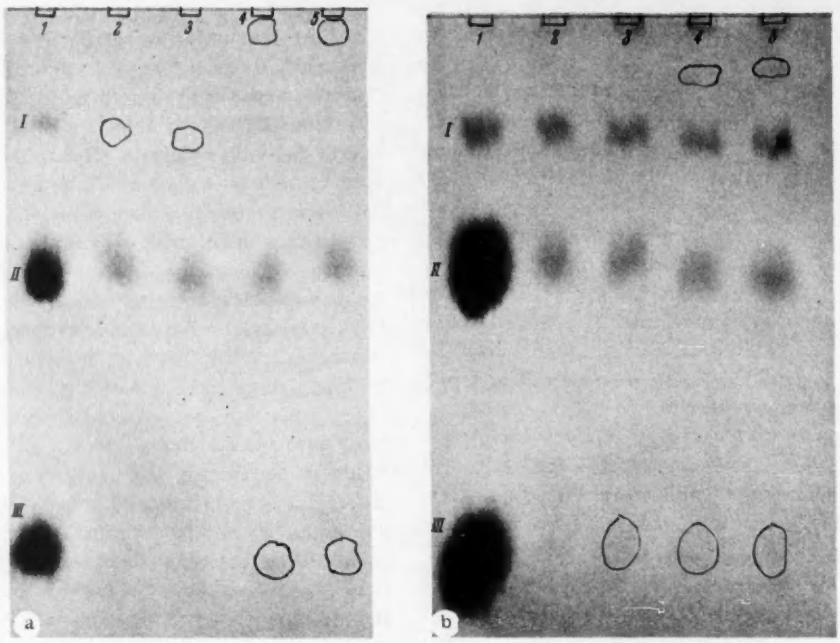

Fig. 2. Chromatographic investigation of the action of transglycosylase on maltose containing trisaccharide (A) and the fraction mixture of trisaccharide and maltose (B). 1) Marker; 2) reaction mixture before incubation; 3, 4, 5) the same after incubation after 2 hours, 1 and 2 days. I) Glucose; II) maltose; III) trisaccharide.

TABLE 1

Action of Transglycosylase on Maltose. Incubation mixture: A) 0.4 ml enzyme + 0.2 ml 3% maltose, pH 5.0; B) 1.5 ml enzyme + 0.75 ml α-dextrin solution + 20 mg glucose + 0.2 ml acetate buffer, pH 5.0

Incubation mixture	Reduction in mg glucose in 1 ml			
	0 hours	2 hours	1 day	3 days
A	6,195	6,200	6,185	6,197
A	6,200	6,220	6,193	6,198
A	6,180	6,190	6,175	6,185
B	7,014	—0,420	—0,420	—

TABLE 2

Action of Transglycosylase on α-Dextrins of Different Sizes. The incubation mixture contained: A) 3.5 ml enzyme + 1.5 ml of solution of α-dextrin + 30 mg glucose + 0.2 ml 1 M acetate buffer, pH 5; B) 1 ml of enzyme + 0.5 ml of 1% starch

Red. of dextrins before/after acid hydrolysis	Composition of dextrins, %		Experiment No.	A – reduction in mg glucose per ml						B – iodine sample	
				extract before acid hydrolysis			extract after acid hydrolysis				
	extract	precipitate		0	30 min	2 hours	0	30 min	2 hours	0	5 days
1 : 3,2	94	5	1	7,860	+0,240	+0,260	8,790	+0,870	+0,870	Violet	Without charge
			2	5,360	+0,460	+0,780	7,275	The same	same		
1 : 5,6	64	36	3	5,220	The same	same	8,340	+0,300	+0,450		
			4	4,260	+0,810	+0,810	8,100	same	same		
1 : 18	16	83	5	5,760	—0,220	—0,750	7,920	+0,220	+0,750		
			6	6,760	—0,980	—0,960	8,760	+0,980	+0,960		

TABLE 3

Effect of Magnesium on the Transglycosylase Reaction. Composition of incubation mixture as in Table 2

Experiment No.	Reduction in mg glucose per 1 ml						Magnesium, mg/ml	
	before acid hydrolysis			after acid hydrolysis				
	0	30 min	2 hours	0	30 min	2 hours	initial court	added
1	5,360	—0,060	—1,180	7,275	The same	The same	6	—
2	5,760	—0,420	—0,750	7,920	" "	" "	6,7	—
3	8,490	—0,690	—0,690	9,660	" "	" "	—	5
4	14,400	—0,510	—0,210	15,300	" "	" "	—	6

TABLE 4

Action of Glucose in the Transglycosylase Reaction on the Average Chain Length of α-Dextrins. Incubation mixture the same composition as in previous experiments

Incubation mixture	Unfermented substance reduction in mg glucose per 1 ml						Ratio of value of reduction before and after acid hydrolysis		
	before acid hydrolysis			after acid hydrolysis					
	0	30 min	2 hrs	0	30 min	2 hrs	0	30 min	2 hours
A with added glucose	1,260	1,393	1,500	5,250	5,407	5,372	1 : 4,16	1 : 3,8	1 : 3,5
	1,584	1,644	2,104	6,195	6,405	6,405	1 : 3,9	1 : 3,8	1 : 3
B without added glucose	0,861	0,861	0,829	3,605	3,710	3,517	1 : 4,18	1 : 4,3	1 : 4,4
	0,448	0,392	0,330	4,872	4,766	4,700	1 : 10,8	1 : 11,6	1 : 14,2

TABLE 5

Action of Transglycosylase and α-Amylase on α-Dextrins. Reducing power of α-Dextrins in mg glucose per 1 ml

Transglycosylase			α-Amylase				
			α- dextrins			starch	
0	30 min	2 hours	0	30 min	2 hours	0	5 min
14,400	—0,510	—0,210	4,033	4,040	4,025	Violet	Colorless
7,680	+0,180	+0,560	8,280	8,300	8,270		
4,230	+0,330	+0,600	8,790	8,800	8,780		

unchanged even after a long interval of time (3 days) and no other spots appear. However, in the mixture of the same enzyme preparation with trisaccharide there is a marked change: a glucose spot appears (Fig. 1, B) which shows that the trisaccharide is split under the action of the enzyme contained in the preparation, showing a substrate-donor for it. The results of these chromatographic investigations, and also of experiments with determination of reduction confirmed our previous conclusion that our isolated liver transglycosylase shows no action on maltose, that is, this disaccharide is not a substrate-donor for it of the glucosyl group. However, as our further experiments showed, in our study of the trans-reaction maltose can play the part of an acceptor, that is, a substance to which the transported substance is transfered. In these experiments the enzyme was incubated with a mixture of maltose and trisaccharide. Thus, in determination of reduction in the experimental mixture of enzyme with a commercial maltose preparation which contained trisaccharide, it was shown that reduction decreased sharply during incubation, which could occur here only if the maltose was not split and its reducing group was blocked by combination with a fragment to form a more complex compound. This idea was confirmed in the chromatographic investigation (Fig. 2, A). We see that after incubation glucose spots appear because of splitting of the trisaccharide, the content of which decreases, and spots of higher molecular weight oligosaccharides (with a small R). An analogous picuture is observed in incubation of the enzyme with fractions which contain a considerable amount of trisaccharide and maltose (Fig. 2, B); and in this case we see that spots of glucose and high molecular weight oligosaccharides appear.

The fact that in the experiments with chromatographically pure maltose there was no splitting of it under the action of liver transglycosylase and in mixture of it with trisaccharide there was a decrease in reduction (this was connected with the appearance of new spots on the chromatogram) indicates that maltose, which is not a substate-donor, can play the part of an acceptor in the trans-reaction being studied.

Experiments with α-dextrins. In studying the action of transglyosylase on dextrins of various sizes, it was shown that this enzyme has a wide range of actions, as Table 2 shows. On the left side of Table 2 there are some data on the analysis of the preparations of α-dextrins used in our experiments; judging by the ratio of reduction before and after acid hydrolysis, they have different average number of chain units and contain different amounts of more or less complex dextrins. Thus, the polysaccharides of preparation No. 1 have an average chain unit of about four glucose residues and contain chiefly oligosaccharides which in alcoholic fractionation appear in the extract, that is, they are low molecular weight carbohydrates. Preparation No. 2 occupies are intermediate position, and preparation No. 3 contains mainly high molecular weight dextrins with a large number of chain units about equal to 18 glucose residues.

The data given on the right side of the table show that when starch is added to the enzyme preparations, the iodine test keeps its color even after five days incubation, which indicates the absence of α-amylase in the enzyme preparations. None the less, as the data in the middle part of the table show, these enzyme preparations show action on dextrins of very different sizes, since here we find changes in reduction which indicate change in size and perhaps in the structure of the dextrins. We see that in one case (Experiments 1 and 2) during incubation, reduction before acid hydrolysis is increased (denoted by the + sign), and in another experiment (Experiments 5 and 6) it is decreased (denoted by the - sign), which is evidently due to reversibility of the reaction. Decrease in reduction during incubation before acid hydrolysis seems especially interesting to us, since it indicates a process of polymerization of glucose or oligosaccharides.

As our following experiments showed, one of the factors which shifts the reaction in just this direction is the magnesium ion. Thus, it was noted that when the enzyme preparation contained a relatively large amount of Mg^{++} (6-7 mg/ml) (Table 3, Experiments 1 and 2), or when this ion was added to the preparation which did not contain it (Experiments 3 and 4), the reaction took place toward polymerization of glucose

or oligosaccharides. It should be pointed out, however, that the addition of magnesium ion in the form of $MgCl_2$ did not always produce the desired result, which indicates the complexity of this mechanism. None the less, these experiments indicate the important role of magnesium in shifting the reaction in the direction mentioned.

Another factor which acts on the direction of the reaction and the formation of oligosaccharides of varying size is glucose, and by its additon to the incubation mixture there can be a decrease in the average chain unit length of the saccharides.

From the data given in Table 4 we see that when glucose is added this value is decreased during incubation, and without glucose, it is increased. These results can be explained thus, that in the presence of glucose the split fragments of dextrin are transferred by the enzyme to glucose and agin the thus formed saccharides become less than the original, while in the absence of glucose part of the transferred dextrins are joined to other dextrins so that the molecules of synthesized saccharides become more than the original.

The studied liver transglycosylase has a very interesting property: it acts on α-dextrins which are resistant to the action of α-amylase. These preparations were obtained by prolonged action of α-amylase on glycogen or starch. The results of these experiments (Table 5) show that the very active α-amylase of saliva which decolorizes starch within five min, has no action on α-dextrins. When these same preparations are incubated with liver transglycosylase there is a definite change in reduction which shows their reorganization. Our later experiments showed that after the action on them of transglycosylase, the α-dextrins undergo splitting by α-amylase (these results will be the subject of a special communication).

SUMMARY

Liver transglycosylase, which has a wide range of actions, catalyzes the reaction of transfer of glucosyl residues of α-dextrins and oligosaccharides of different sizes, beginning with trisaccharides and ending with high molecular weight dextrins. Maltose is not a substrate-donor but can be a cosubstrate-acceptor in this transfer reaction.

The studied enzyme has the property of changing the size of the average chain length of the dextrins and of causing a change of α-dextrins which are resistant to the action of α-amylase.

We have described some characteristics of the transfer reaction in the presence of magnesium ion and glucose.

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THE SIGNIFICANCE OF PROTEIN FOR THE INFECTIOUSNESS OF UREA TREATED BACTERIOPHAGE

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In the solution of the question of the function of nucleic acids an important role is played by investigations carried out on viruses. The chief value of the results is that purified RNA isolated from tobacco mosaic virus and other objects can induce in host cells the development of full virus particles. However, with respect to viruses with more complex structures which contain DNA, such clear results have not been obtained up to now. In the case of bacteriophage indirect data have already shown well enough that the reproduction of new virus particles cannot occur without the participation of DNA. However, on the other hand, in natural infection of bacterial cells there occurs in phage along with the DNA a small amount of protein which is contained within the phage corpuscles [6]. The necessity and function of a separate phage protein in the infection of cells has not been sufficiently explained. For a solution of this question it is necessary to investigate the infectious activity of phage particles as they are destroyed to varying extents up to full liberation of DNA from the protein.

The process of infection of bacteria consists of two main steps. First there should be penetration of the active principle from the phage into the bacterium through the little permeable cell wall and cytoplasmic membrane. Then begins the actual action of the virus within the cell.

The first step, adsorption of the phage on the bacterium, requires preservation of its cell wall and of the protein covering of the phage. After destruction of these structures, later adsorption of the phage and hence the whole cycle of its development becomes impossible. However, the ability of such a phage to react with bacteria can be restored if protoplasts are used as recipients, that is, bacteria, deprived of cell walls [11]. It is known that inside the protoplasts there can be penetration of macromolecular particles (DNA) which do not have the special abilities of the whole phage particles, which, for example, can produce local lysis of the cell walls [3]. Protoplasts are a system suitable for experiments with phages since it has been shown that in the protoplasts there can occur full development of normal virus particles. [1, 8]. Data given in the literature indicate that in some cases partly destroyed phage corpuscles which have lost the ability to infect whole cells can infect protoplasts. Spizizen [10] destroyed phage T2 by osmotic shock and observed that the virus then ceased to infect whole cells, but caused the development of normal phage in protoplasts. A study of the properties of preparations of damaged phage allowed the explantation that the infectious activity depended on the remaining incompletely destroyed particles in which DNA was bound to protein.

Fraser et al., [4] treated phage T2 with a concentrated solution of urea and also obtained a preparation inactive with respect to whole bacteria but causing development of phage in protoplast obtained under the influence of lysozyme. On the basis of the data of inactivation of the resulting preparations by deoxyribonuclease (DNA-ase), the authors concluded that infectious activity of their preparation was connected with DNA freed from the protein envelope of the corpuscles by the action of urea.

We studied the functional role of the protein in DNA of bacteriophage and obtained results which indicated that infectiousness of phage treated with urea was not connected with its free DNA, but depended on the presence in these preparations of damaged phage particles which were inactive toward intact bacteria. Therefore phage treated with urea could be a material favorable for studing the question of the roles of various components, including the proteins which are found in the virus composition and are needed for infecting the cells. In this connection we undertook a detailed study of the parallels of infectious activity, chemical composition, and morphological structure of phages treated with urea.

METHODS

In the work we used the following intestinal-dysentery phages: T4r, known from the literature, obtained from Prof. F. Gerchik (Czechoslovakia); DM, isolated from the soil by T.N. Broker, similar in biological properties and chemical composition to T phage (in

particular, DM contains 5-hydroxymethyl cytosine); H2, presented by F.I. Ershov (2nd Moscow State Medical Institute). The bacterial protoplasts were obtained with lysozyme on the basis of the method of Repaske [9]. For this purpose we used: E. coli B, known from the literature, sensitive to phages T4r and DM, but resistant to H2; and Sh. dysenteriae Newcastle, obtained from F.I. Ershov, typical in properties and sensitive to phages T4r, DM, and H2.

For the preparation of protoplasts we used six hour broth cultures which contained $5\text{-}6 \cdot 10^8$ cells/ml. The cultures were concentrated by centrifuging and resuspended in 0.05 M tris buffer (trishydroxymethylaminomethane), pH 7.9, such that the final cell concentration was $5\text{-}6 \cdot 10^9$ per ml. In this medium the bacteria were treated with lysozyme in a concentration of 100 μg/ml in the presence of 0.5 M sucrose and versene (108μg/ml). Formation of protoplasts was controlled in a phase contrast microscope; it was usually complete in 5-10 min. The amount of bacteria remaining which were still capable of forming a colony under ordinary conditions of seeding did not surpass 2% for cultures of E. coli B and Sh. dysenteriae Newcastle. For stabilization of the resulting protoplasts we added serum albumin to a final concentration of 2%. Then the protoplasts were precipitated by centrifuging and resuspended in broth with 0.5 M sucrose and 2% serum albumin. The final concentration was $5 \cdot 10^8$ to $1.0 \cdot 10^9$ protoplasts/ml.

Concentrated phages T4r and DM were obtained from lyzates of E. coli B, and of H2 from Sh. dysenteriae Newcastle. We used a synthetic Adams medium with addition of 100 μg/ml of tryptophan and 1/10 the volume of meat peptone broth. The average phage titer was $5 \cdot 10^9$ corpuscles/ml. One liter of such lyzate was freed from bacteria by centrifuging at 3000 rpm for 30 min and then cooled to 0-4° and precipitated by $(NH_4)_2SO_4$ at 0.4-0.6 saturation. The lyzate with the ammonium sulfate remained over night at 0°. The precipitate was separated by centrifuging at 2500 rpm for 15 min and was resuspended in 10 ml of synthetic Adams medium. The resuspended precipitate which contained most of the phage from the lyzate was freed from large particles by centrifuging at 3000 rpm for 5 min. The supernatant liquid was submitted to rapid centrifuging at 14,000 to 15,000 rpm for 45 min. The resulting phage precipitate was again resuspended in 1 ml of synthetic Adams medium and purified by centrifuging at 3000 rpm for 5 min. The supernatant liquid usually contained 10^{12} corpuscles per ml. The suspension of concentrated phage was treated with an 8 M solution of urea in 0.1 M NaCl as described by Fraser et al., [4]. As preliminary experiments showed, the phages which we used were characterized by different sensitivities toward urea. Therefore the duration of treatment of the concentrated phages varied from 30-90 min. The phage treated with urea was dialyzed against 0.1 M NaCl for 18 hours at 4-6° with two changes in NaCl solution.

RESULTS AND DISCUSSION

After the treatment with urea, active virus particles were not observed in the phage suspension when it was studied by the method of agar layers [5] in an indicator strain. Therefore we ran experiments to show whether the phage preparations treated with urea had the ability to infect protoplasts.

For this purpose, 0.5 ml of protoplast was mixed with 0.5 ml of the phage preparation treated with urea, first diluted 100-200 times by broth with sucrose and albumin. In parallel experiments the same amount of preparation was mixed in the presence of sucrose and albumin with 0.5 ml of 4 hour broth culture which contained whole cells. The experiment was also accompanied by controls for accidental presence of phage in the medium for the protoplasts; in all the experiments the control seedings gave negative results.

The suspensions of bacteria or protoplasts with the phage preparations were incubated at 37° for 10 min. Infectious activity in the studied preparations was determined by the method of agar layers on a number of negative colonies. The results of the experiments are summarized in Table 1.

TABLE 1

Infectious Activity of Intact and Urea Treated Phage with Respect to Whole Cells and Protoplasts

Object of study	T4r		DM		H2	
	intact	urea treated	intact	urea treated	intact	urea treated
E. coli B.						
Whole cells	$5 \cdot 10^{11}$	0	$2 \cdot 10^{11}$	0	0	0
Protoplasts	—	10^6	—	10^6	—	$5 \cdot 10^5$
Sh. dysenteriae Newcastle						
Whole cells	$5 \cdot 10^{11}$	—	$2 \cdot 10^{11}$	0	$3 \cdot 10^{11}$	3
Protoplasts	—	—	—	10^6	—	$5 \cdot 10^5$

It follows from the results in Table 1 that the phage preparations retain activity toward protoplasts and are entirely inactive toward intact cells. The retained activity shown only toward the protoplasts varied depending on the medium and in the presence of meat peptone broth and albumin was 0.0001% of the original phage titer, and in the Adams medium with albumin, about 0.00001%. The infectiousness of the phage treated with urea was shown not only toward protoplasts obtained from bacteria which were sensitive to the original phage, but also toward protoplasts of strains whose whole cells were not infected by the original phage (E. coli B and phage H2). Evidently the removal of the surface structure of the bacteria during its treatment of lysozyme eliminated the difference between phage sensitive and phage resistant strains. It is probable that this occurs if the resistance is determined by an external barrier to the phage depending on a receptor apparatus of the bacteria.

This finding agrees with the data of Spizizen on the ability of phage which has undergone osmotic shock to infect protoplasts obtained from resistant strains. The infection of resistant strains by phage preparations which had been treated with urea is further evidence that infectiousness in this case does not depend on the possible presence in the preparations of a residual amount of whole phage particles.

To explain the nature of the infectiousness of the urea treated phage toward the protoplasts we can make two suggestions: 1) Infectiousness is determined by the DNA of the phage, liberated by the urea treatment; 2) infectiousness depends on the presence of damaged but not completely destroyed phage particles which do not act on intact bacteria but are active toward protoplasts.

For a test of the first suggestion we must establish whether the DNA is liberated in treating the phage by urea and whether the infectiousness of the preparation depends on the amount of free DNA.

For this purpose we determined spectrophotometrically (by absorption at 260 mμ) the amount of DNA in the following samples: a) In intact phage after incubating it with deoxyribonuclease (DNA-ase) (100 μg/ml at 37° for 60 min); b) in intact phage, incubated without DNA-ase); c) in phage submitted to the action of urea and then treated with DNA-ase; d) in phage submitted to the action of urea and incubated without DNA-ase. In all these samples we determined both the total absorption and also the absorption of fractions of acid soluble nucleotides before and after incubation. For this purpose the unsplit RNA in the sample was precipitated by an equal volume of 6% perchloric acid in the cold and after one hour the precipitate was removed by centrifuging. The quantity of nucleotides observed in the centrifugate after incubation corresponds to the amount of free DNA obtained by the action of DNA-ase.

As these experiments showed (Table 2) practically all the DNA in intact phage is protected from DNA-ase, while in the preparations treated with urea an average of 74% of the DNA is found in the free state, split by DNA-ase. However, the infectiousness of the preparation of phage treated with urea, in which the free DNA had been split out by the enzyme, was not markedly lowered. Also, the activity of the intact phage was not changed by treatment with DNA-ase.

These experiments showed that the infectiousness of our preparations did not depend on the free DNA of the phage which separated from its particles under the influence of urea. Hence, the ability to infect protoplasts can depend only on those elements which are contained in the preparation after treatment with urea, in which the DNA remains included in the protein fragments. The latter protect DNA from the action of DNA-ase. On the basis of these results we can conclude that for infectiousness we must have a complex of DNA with protein. This conclusion was confiremed in the following experiments. It was shown that deproteinization of a phage preparation treated with urea by phenol saturated with water or by a mixture of chloroform and octyl alcohol resulted in complete loss of infectiousness, although the DNA was not thus removed from the solution. On the other hand, it is known well enough

TABLE 2

Effect of Deoxyribonuclease on the DNA Content in Intact Phage and in Phage Treated with Urea

Object studied	Infectiousness, %		Amount of DNA, %	
	T4r	DM	T4r	DM
Intact phage after incubation without DNA-ase	100	100	100	—
Intact phage after incubation with DNA-ase	100	102	95	—
Phage treated with urea and incubated without DNA-ase	100	100	100	100
Phage treated with urea and incubated with DNA-ase	95	94	25	27

that isolation of the protein component of the phage also does not lead to infection by it. Hence, the infectiousness of the phage preparation treated with urea is determined by the complex of DNA with protein.

Further experiments were carried out to explain the nature of this complex. Chiefly we studied the immunological properties of the protein which occurred in its composition.

The infectious activity of the urea treated phage preparations, like that of the original phage, suppressed the antisera obtained in immunizing rabbits with the corresponding intact phage (Table 3). The specificity of the observed neutralization was confirmed by central experiments with normal rabbit serum. Evidently in great measure one of the proteins found in the active complex is an antigen localized along with other proteins in the tail part of the whole phage corpuscle. This appears since the reaction of neutralization of the serum when urea treated phage acts on the preparation does not depend on the presence of the complement which is required, as is known, for the reaction with antigen from the head of the phage.

In order to obtain the idea of the dimensions of the active particles in the phage preparation treated with urea, the latter was centrifuged at 14,000 rpm for 50 min at the same time as the suspension of original phage, after which we determined the activity of the precipitate and the supernatant liquid. The same results were obtained in centrifuging the whole phage and the preparation after the action of urea: in both cases 80-90% of the activity passed into the precipitate.

The data on distribution of activity in the centrifuged preparation agreed with the results of experiments in which we studied under the same conditions the ratio between DNA and protein in the centrifugate and the precipitate (Fig. 1).

In Fig. 1 we give the absorption spectrum (in % of maximum) of the original preparation of phage T4r treated with urea, the supernatant liquid, and the precipitate. All three curves have absorption maxima characteristic of DNA. However, in the region 240-250 mμ and 280-290 mμ the absorption of the precipitate

TABLE 3
Effect of Antiphage Serum on Infectiousness of Intact Phage and Phage Treated with Urea

Object of study	infectiousness, %	
	T4r	DM
Intact phage + normal serum	100	100
Intact phage + antiphage serum	0	0
Phage treated with urea + normal serum	100	100
Phage treated with urea + antiphage serum	0	0

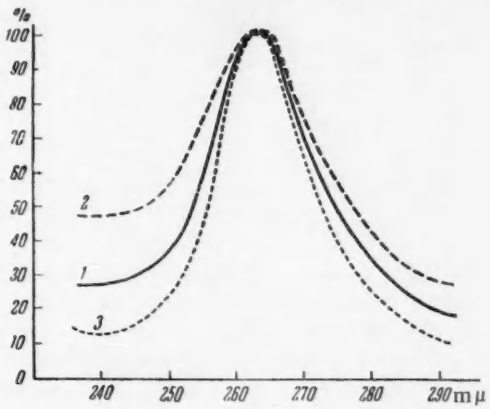

Fig. 1. Absorption spectrum in the ultraviolet, expressed in % of maximum. 1) Original preparation of phage T4r treated with urea; 2) precipitate after centrifuging this preparation; 3) supernatant liquid after centrifuging this preparation.

is considerably greater than the absorption of the supernatant liquid. Hence, there is relatively more protein in the precipitate and relatively more DNA in the supernatant liquid. The initial preparation occupies an intermediate position. This shows that the active principle in the preparation of phage treated with urea which reproduction of the phage in the protoplast consists of DNA and protein which form the corpuscle whose dimensions do not differ appreciably from those of the particles of

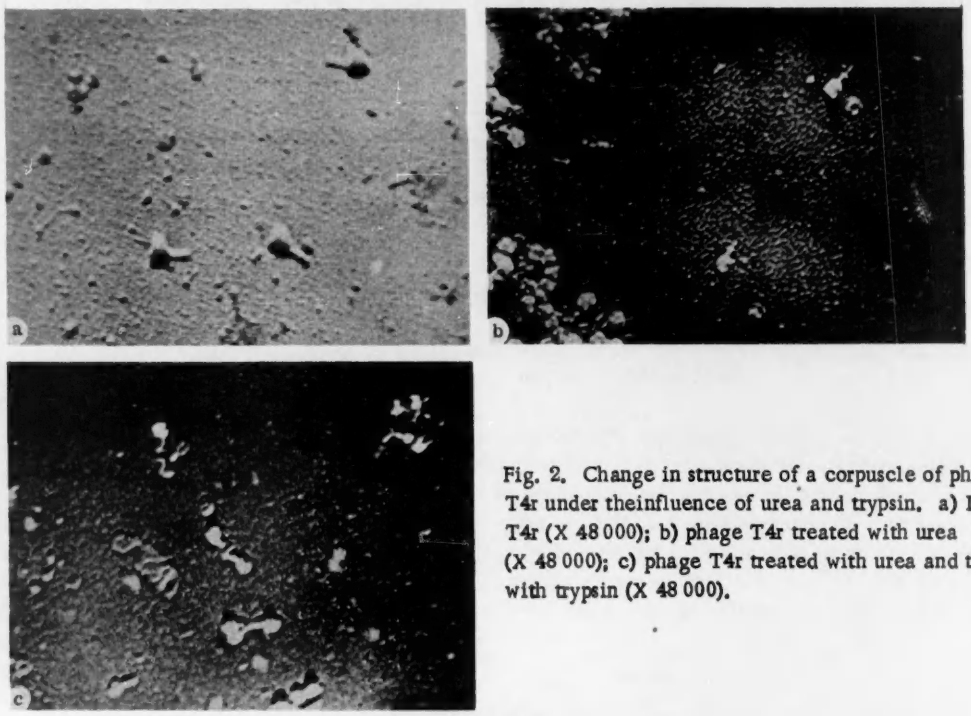

Fig. 2. Change in structure of a corpuscle of phage T4r under theinfluence of urea and trypsin. a) Intact T4r (X 48 000); b) phage T4r treated with urea (X 48 000); c) phage T4r treated with urea and then with trypsin (X 48 000).

intact phage. Therefore, for further study of the nature of the active particle it was expedient to use the electron microscope. The preparation for the electron microscope was made by the method of dropping dialysis ona collodian film [2] dried in air and shadowed with chromium. Microscopy was carried out in a Tesla electron microscope.

Fig. 2 shows the structure of intact phage (a) and phage treated with urea (b). Treatment with urea evidently destroys the structure of the distal end of the tail of the phage particle which explains its loss of ability to be adsorbed on intact bacteria. Under the influence of urea the outer covering of the tail is destroyed and as a result the inner axis of the protein part is partially exposed. It is known that such an exposure of the protein axis is also caused by other chemical agents such as cadmium salts, hydrogen peroxide, etc. [7].

From the point of view of the problem of the function of the separate protein component of phage of its infectiousness, it is interesting to consider whether inactivation of the urea treated phage preparation occurs by destruction of the axis of the protein tail exposed by the action of this reagent. It is known that the outer protein which makes up the covering of the phage and the intact phage itself are not harmed by the action of trypsin. After urea treatment one of the inner proteins of the corpuscle, the protein axis of the tail, is observed. It can be assumed that under the action of trypsin on the whole phage corpuscle the protein axis of the tail is not destroyed because it is protected by a covering of another protein which is reisistant to trypsin. Therefore we carried out experiments on the effect of trypsin on infectious activity and on the morphology of the particles which occur in the phage suspension after treatment by urea.

The urea treated phage preparation was incubated for one hour at 37° with different concentrations of crystallin trypsin (from 20 to 0.02 μg) in 0.1 M NaCl. Control samples were incubated with NaCl without trypsin. After the end of incubation action of trypsin on the sample was stopped by 100 fold dilution with broth with 0.5 M sucrose and 2% serum albumin. The resulting mixture was studied with protoplasts. Thus the incubated mixture contained a preparation of destroyed phage which had first been treated with trypsin, protoplasts, and trypsin with the highest final concentration 0.2 μg/ml.

As control experiments showed, trypsin in such concentrations does not destroy protoplasts and does not hinder the appearance of infectious activity of phage preparations during their incubation with protoplasts. In these experiments we also studied the effect of trypsin on a suspension of intact phage whose titer was determined by whole cells.

TABLE 4 Effect of Trypsin on Infectiousness of Phage T4r Treated with Urea

Object of study	Trypsin concentration, μg/ml	Infectiousness, %
Intact phage	0	100
	20	115
Phage treated with urea	0	100
	0,02	91
	0,2	72
	2,0	8
	20,0	4

The resulting data given in Table 4 indicate that, as distinct from intact phage, the virus treated with urea is inactivated by the action of trypsin. This can be explained in two ways: Under the influence of urea there is a change in the structure of the outer protein of the phage and it becomes sensitive to an enzyme, or there is later destruction of the exposed axis of the tail protein, which is normally sensitive to proteases but is usually covered by another resistant protein. Preliminary electron microscope study showed that the second assumption is true. When trypsin acts on phage which has first been treated with urea, the structure of the head of the corpuscle undergoes no visible change (Fig. 1, c). At the same time, trypsin destroys the exposed axis part of the tail. Hence, the protein which composes it is sensitive to protease and differs in this respect from the outer protein of phage. These results also indicate that the axis protein of the tail is necessary for infectious activity of the phage preparation toward protoplasts. Hence we can conclude that the axis protein of the tail is one of those chemical components of phage particles which are directly required for theinfection of bacteria by the virus.

In this connection we can suggest that the axis protein penetrates into the bacteria along with DNA. We note that this protein differs from the outer protein of phage in its sensitivity to the proteolytic enzyme, trypsin. It is still possible that the axis protein assures the introduction of DNA into the cell (protoplast) and then is destroyed the intracellular proteases.

SUMMARY

When large intestinal dysentery phages T4r, DM, and H2 are treated with 8 M urea solution they gave preparations which are inactive toward intact cells but can infect bacterial protoplasts.

DNA liberated by treatment of the phage with urea does not cause the activity of the preparations since the action of DNA-ase is accompanied by destruction of free DNA without decrease in activity of the preparations. Removal of protein from the preparations by phenol or chloroform results in loss of activity. Antiphage serum obtained by immunization of rabbits with intact phages also removes the activity of the preparations. The active principle of the phage preparations precipitates with centrifuging at the same rate and in the same time as in the intact phage.

Electron microscope study of the urea treated phage preparations shows the presence of phage corpuscles with exposed inner axis at the distal end of the tails, which is destroyed by the action of trypsin with loss of infectiousness of the preparation.

The results indicate that the activity of urea treated phage preparations depends on partly destroyed phage corpuscles which do not react with intact cells because of damage to the distal ends of the tails. The protein of the axis portion of the latter is necessary for infectiousness of the phage.

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QUANTITATIVE PAPER CHROMATOGRAPHY OF HIGHER FATTY ACIDS IN THE FORM OF METHYL ESTERS ($RCOOC^{14}H_3$)

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Paper chromatographic separation of higher fatty acids has become possible only with the introduction into chromatographic analysis of the method of the "reverse phase". [1]. According to this method the paper is impregnated with some hydrophobic substance (rubber, paraffin, naphthalene, kerosene, etc.). On the paper thus rendered hydrophobic separation of the higher fatty acids occurs because of a continuous separation between the hydrophobic and hydrophilic phases as the system of solvents moves on the chromatogram.

The difficulty with the existing methods of separating the higher fatty acids [2, 3] is the impossibility of simultaneous separation on one chromatogram of the so-called "critical pair" of fatty acids. Thus the saturated palmitic acid (whic contains 16 carbon atoms) cannot be separated on the chromatogram from oleic acid (which contains 18 carbon atoms and has one double bond). The R_f of saturated stearic acid, which contains 18 carbon atoms, corresponds to the R_f of eicosenic acid which contains 20 carbon atoms and one double bond. In many oils, acids forming "critical pairs" are found together. In the suggested methods [2,3] mixtures of higher fatty acids were studied by chromatographic separation and then the unsaturated fatty acids were oxidized and the remaining unreacted saturated acids were chromatographed. Comparison of the chromatograms indicated the compositon of the higher fatty acids in the samples studied. Thus the impossibility of separating the "critical pairs" of higher fatty acids on one chromatogram is one of the difficulties in the use of this method for exact quantitative chromatography of any mixture of higher fatty acids.

In any event, the most effective method for identification of spots of the higher fatty acis on the chromatogram by silver and bismuth salts [4] does not have a high resolving power (the lower limit of sensitivity of the method averages 10 μg). Direct photometric examination of the chromatogram can be used for quantitative analysis with identified spots of the higher fatty acids, but the accuracy of this method is not high enough.

Since the separation of the higher fatty acids is difficult, attempts have been made to chromatograph derivatives of the higher fatty acids, especially the esters. Introduction of the ethyl group permitted Boldingh [5] to separate "critical pairs" of higher fatty acids: to show the esters the chromatogram was immersed in an alcoholic solution of a lipophilic dye, Sudan IV. However, the use of a lipophilic dye for identification of higher fatty acid esters on the chromatogram has little promise, since the ester molecule in reacting with the lipophilic dyes, especially Sudan IV, gives compounds of an adsorptive character. Also, rendering the paper on which the chromatogram is developed hydrophobically leads to adsorption of the the hydrophobic dyes.

Thus, in working out a method for quantitative paper chromatography of the higher fatty acids we must be able to separate the "critical pairs" of higher fatty acids and find a sufficiently sensitive method for accurate quantitative determination of the separate acids on the paper after the corresponding chromatographic separation.

The difficulties which arise in working out a method for quantitative chromatography of the higher fatty acids can be overcome by introducing into the carboxyl of the analyzed mixture of higher fatty acids a radical which contains a radioactive isotope. The radioactive radical introduced into the compounds being studied for the purpose of quantitative chromatography should satisfy the following conditions:

1) The reaction with the radioactive compound should be quantitative in all components of the mixture under mild conditions of synthesis; 2) the excess radioactive compound used for introducing the labeled atom should be easily removed, as well as any possible side products of the synthesis.

In our work we chose the method of separation and quantitative determination of higher fatty acids in the form of methyl esters labeled with radioactive carbon, C^{14}, in the methyl group in a reverse phase chromato-

graphic system followed by radioautography of the chromatogram. For methylation of the higher fatty acids we used radioactive diazomethane, $C^{14}H_2N_2$.

$$R \cdot COOH + C^{14}H_2N_2 \rightarrow R \cdot C \cdot OO \cdot C^{14}H_3 + N_2.$$

This reaction goes at room temperature in a neutral medium (ether) in the absence of acid or alkaline condensing media or catalysts, which excludes any possible thermal, acid, or oxidative (by atmospheric oxygen) isomerization of the products of the synthesis. Usually such a method of methylation is employed for obtaining methyl esters of labile acids [6]. Carrying out the synthesis does not meet any difficulties from the point of view of method and can take place with very small amounts of material.

With some excess of diazomethane the methylation reaction occurs quantitatively for the higher fatty acids and gives practically no byproducts other then methyl alcohol which can occur as the result of decomposition of diazomethane under conditions of prolonged standing of the reaction mixture at room temperature. The excess unreacted radioactive diazomethane is easily removed by passing nitrogen or air through the reaction system and repeated washing of the synthesized methyl esters of the higher fatty acids with ether and subsequent removal of the ether from the reaction system by a stream of nitrogen or air.

The methyl esters of the higher fatty acids are separated on hydrophobic chromatographic paper. At the same time we carry out chromatography of the labeled methyl ethers of individual fatty acids which permits identification in the analyzed samples. The introduction of the methyl group in the higher fatty acids permits chromatographic separation of "critical pairs" of higher fatty acids

Radioautographs are obtained from the chromatograms; starting from the intensity of the darkened spots on the radioautographs and considering the differences in molecular weights of the acids, the absolute content of the various acids is determined. The sensitivity of the method depends on the specific radioactivity of the methyl esters of the higher fatty acids studied, the sensitivity of the X-ray film and the length of keeping the chromatogram in contact with the X-ray film. Changes in all these factors can result in a very high separating power of the suggested method.

METHODS

Obtaining a preparation of higher fatty acids. Oil from the seeds of the castor plant (Ricinus communis) and the peanut (Arachis hypogea) was extracted with dry ether in a Soxhlet apparatus for 15 hours. At the end of the extraction the ether was distilled off on the water bath. Saponification of the oil and obtaining the preparation of higher fatty acids was carried out as described by Vereshchagin [3]. As "indicators" in the chromatographic study we used individual fatty acids or mixtures of acids which were also methylated with radioactive diazomethane.

Synthesis of methyl esters of higher fatty acids. Radioactive diazomethane ($C^{14}H_2N_2$) for the synthesis of the methyl esters of higher fatty acids was obtained from nitroso methyl-C^{14}-urea, $C^{14}H_3N(NO)CONH_2$, synthesized from methyl-C^{14} amine hydrochloride ($C^{14}H_3NH_3Cl$). The synthesis of nitrosomethyl urea was carried out by the method of Arndt [7].

From 241 mg of methyl-C^{14} amine hydrochloride (activity 0.2 μcurie)we synthesized 150 mg of nitroso-methyl-C^{14} urea with specific activity 0.63 μcurie/g. The preparation was kept in a vacuum desiccator at temperatures of 0 to 5°.

The synthesized nitrosomethylurea was used for obtaining labeled diazomethane by the method suggested by Arndt [8]. The reaction occurs by the following scheme:

$$C^{14}H_3N[NO]CONH_2 + KOH \rightarrow C^{14}H_2N_2 + KCNO + H_2O.$$

Decomposition of nitrosomethyl-C^{14} urea and synthesis of the methyl esters of the higher fatty acids took place in the apparatus (Fig. 1) which consisted of a distillation apparatus of small size with sealed on stopcocks which permitted passageof a stream of nitrogen or air through the system.

In flask 1, volume 3-5 ml, we placed about 10 mg of nitrosomethyl-C^{14} urea and 0.6-1.0 ml of dry ether, and in another flask, 2, with the same volume, 5-6 mg of higher fatty acids, accurately weighed on an analytical balance, and 0.3 ml of dry ether. Flask 2 was shaken gently to dissolve the acids in the ether and joined to the apparatus (Fig. 1). Flask 1 was cooled to 0°, and

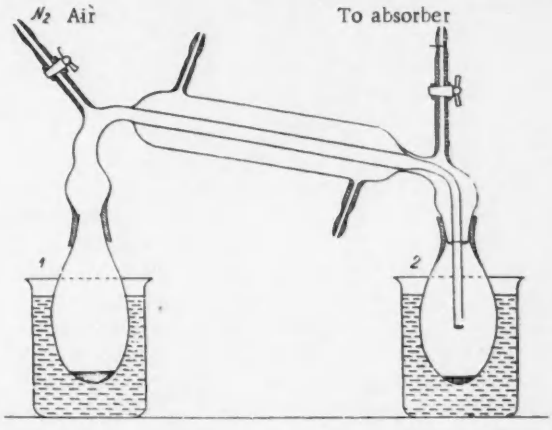

Fig. 1. Apparatus for synthesis of methyl esters of higher fatty acids.

0.2-0.3 ml of 40% KOH in ethanol, also cooled to 0°, was quickly poured in and the flask was at once joined to the apparatus. The temperature in flask 1 was kept at 3 to 5°, and to shake the contents of the flask the

whole apparatus was rocked slightly by moving the support to which it was held with a plastic pad. After 5-7 min the decomposition of the nitrosomethyl-C^{14} urea was complete. The apparatus was fastened and water was passed into the condenser. Then flask 2 was cooled with a mixture of ice and Na Cl and flask 1 was placed in a beaker of water heated to 50°. First the stopcock leading to the abosorber was opened; the absorber consisted of three Tishechenko bottles, the first of which was filled with a solution of higher fatty acids in alcohol, the second with a solution of benzoic acid in ether, and the third with water. In the absorber there occurred combination of the small amounts of diazomethane and methanol which might be formed in minute amounts by decomposition of diazomethane. Diazomethane distilled with the ether into flask 2, where the synthesis of the methyl ethers of the higher fatty acids occurred with evolution of nitrogen. The stopcock which connected flask 2 with the absorber was closed, and the apparatus with closed stopcock stood for 3-4 hours at room temperature for full methylation of the higher fatty acids.

As a sign that the reaction was complete a yellow color appeared in the reaction mixture which was due to unreacted excess diazomethane. At the end of the synthesis a stream of nitrogen or air was passed through the system to remove unreacted diazomethane and ether. For complete removal of traces of diazomethane 1.0 ml of absolute ether was three times added to the flask 2 with subsequent removal by passing nitrogen or air through the system.

Making the paper hydrophobic and carrying out the chromatographic separation. For treating the paper we used medical vaseline, which is a homogeneous greasy mass of a white color and without odor. The dry residue after burning and igniting a sample of vaseline was about 0.2%. A strip of paper* 2.0 x 55.0 cm in size was immersed in a 5% solution of vaseline in carbon tetrachloride and suspended in a horizontal position. We could use benzene or ligroin as the solvent, but carbon tetrachloride dissolved vaseline better and more quickly volatilized from the paper, which decreased the downward flow on the paper and gave a more event treatment by the vaseline. The strip of paper was weighed before and after treatment with vaseline and for the chromatographic study we used hydrophobic paper containing 8-12% vaseline. In running comparative studies (determination of Rf of higher fatty acids from the mixture being studied and the parallel samples) it was best to choose strips of paper with a variation in vaseline content not more than 1%.

We place from 1 to 30 mg of the studied mixture of methyl esters of higher fatty acids on the strip of hydrophobic paper at a distance of 6 cm from the edge, using a micropipet. The amount of substance deposited was more accurately measured by the radioactivity of spots with an end counter.

We will give the calculation of absolute amount of higher fatty acids for one of the experiments. For the synthesis of methyl esters we took a weight of 5.1 mg of higher fatty acids from peanut oil. The synthesized esters were dissolved in 1 ml (exactly) of acetone and several spots of 0.005 ml of solution, corresponding to 25.5 μg of higher fatty acids were deposited on the hydrophobic chromatographic paper. The average radioactivity of the spots was 388 counts/min (without introducing a correction for the effectiveness of counting of the end counter MST-17). In later deposits of the higher fatty acid solution of a given sample on the chromatogram we determined the amount of higher fatty acids more accurately by the radioactivity of the spots, since there was a direct proportionality between the radioactivity of the spots and the quantity of methyl esters of higher fatty acids which they contained.

After determining the radioactivity of the spots the strip of chromatographic paper was placed in a chamber for descending chromatography. We used the following systems of solvents: acetic acid-acetone (3:1); methanol-acetone (2:1 or 3:1); and ethanol-acetone (2:1). The ratio of solvents is given by volume. Chromatographic separation was carried out for 14-16 hours at room temperature without reaching outflow of solvent from the paper. In the system acetic acid-acetone the process of separation was carried out for a longer period of time (up to 36 hours).

Radioautographs of the chromatograms, photometrometry on X-ray film, and treatment of the results. The dry chromatogram was enclosed in a casette with X-ray film for five to seven or more days depending on the activity of the original spot on the chromatogram. It was necessary to consider that in the mixture studied the separate higher fatty acids might be present in very slight amount and therefore part of the chromatogram was enclosed in the casette with the X-ray film for a longer time. In comparative quantitative study of the preparations of higher fatty acids and identification of the separate acids by "indicators" the corresponding chromatograms were enclosed in casettes in contact with one X-ray film. In the present work we used X-ray film X for exposure with intensifying screen sensitivity 150 and 260 inverse roentgen, and Agfa-Routgen-Duro film for medical exposure (produced in Eastern Germany). The films were treated in the dark.

The radioautographs were subjected to photometric measurement in an MF-4 registering microphotometer, in the construction of which some changes were made. The illuminating objective was removed and the projecting objective was replaced by a slit 1.2 mm wide. The use of two slits, measuring, and a supplementary one, permitted separation from the wide beam of parallel rays of a beam in the form of a rectangle with wide 1.2

* In this work we used slow filtration chromatographic paper from the Volodar Leningrad Factory.

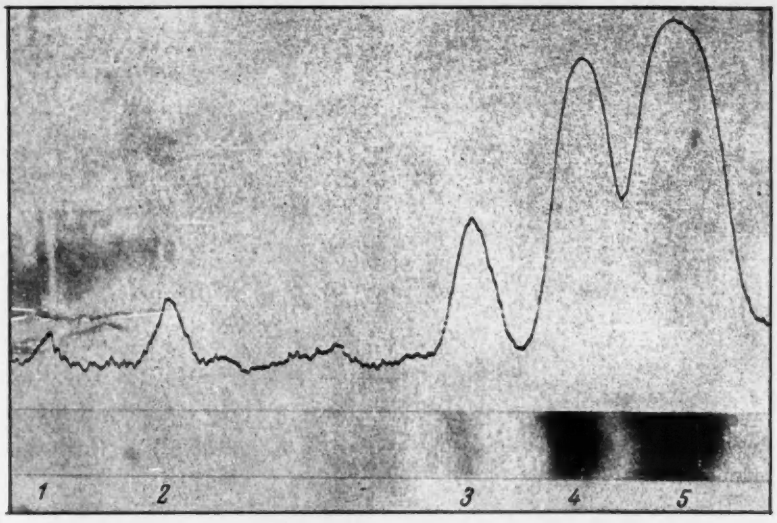

Fig. 2. Radioautograph from the chromatogram of the mixture of methyl esters of higher fatty acids and corresponding photometric curves. Solvent system: Acetic acid-acetone (3:1). Acids: 1) Lignoceric; 2)behenic; 3) palmitic; 4) oleic; 5) linoleic. Deposited on the chromatogram, 25.03 μg of mixed acids.

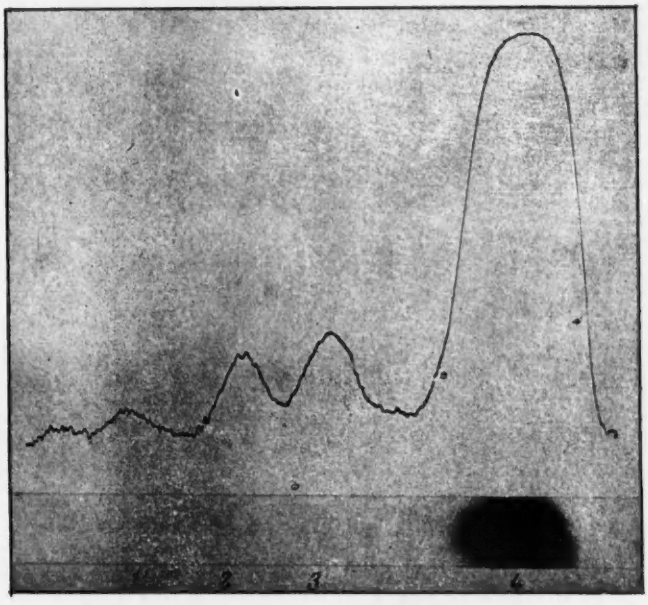

Fig. 3. Radioautograph from the chromatogram of methyl esters of higher fatty acids of castor oil and the corresponding photometric curve. Solvent system: Acetic acid-acetone (3:1). Acids: 1) stearic; 2) oleic; 3) linoleic; 4) ricinoleic. Deposited on the chromatogram, 24.3 μg of the studied mixture of acids with radioactivity 453 counts/min. Time of separation 26 hours. Chromatogram kept in contact with X-ray film 15 days.

mm and hieght 20 mm; this permitted photometric study of a roentgenogram with a width of 20 mm.

A test of the microphotometric study of the radioautographs with width 20 mm on the MF-4 microphotometer with slit width 4 mm followed by summation of the series of resulting curves and the suggested method of single photometric study of the radioautographs in the same apparatus gave concordant results.

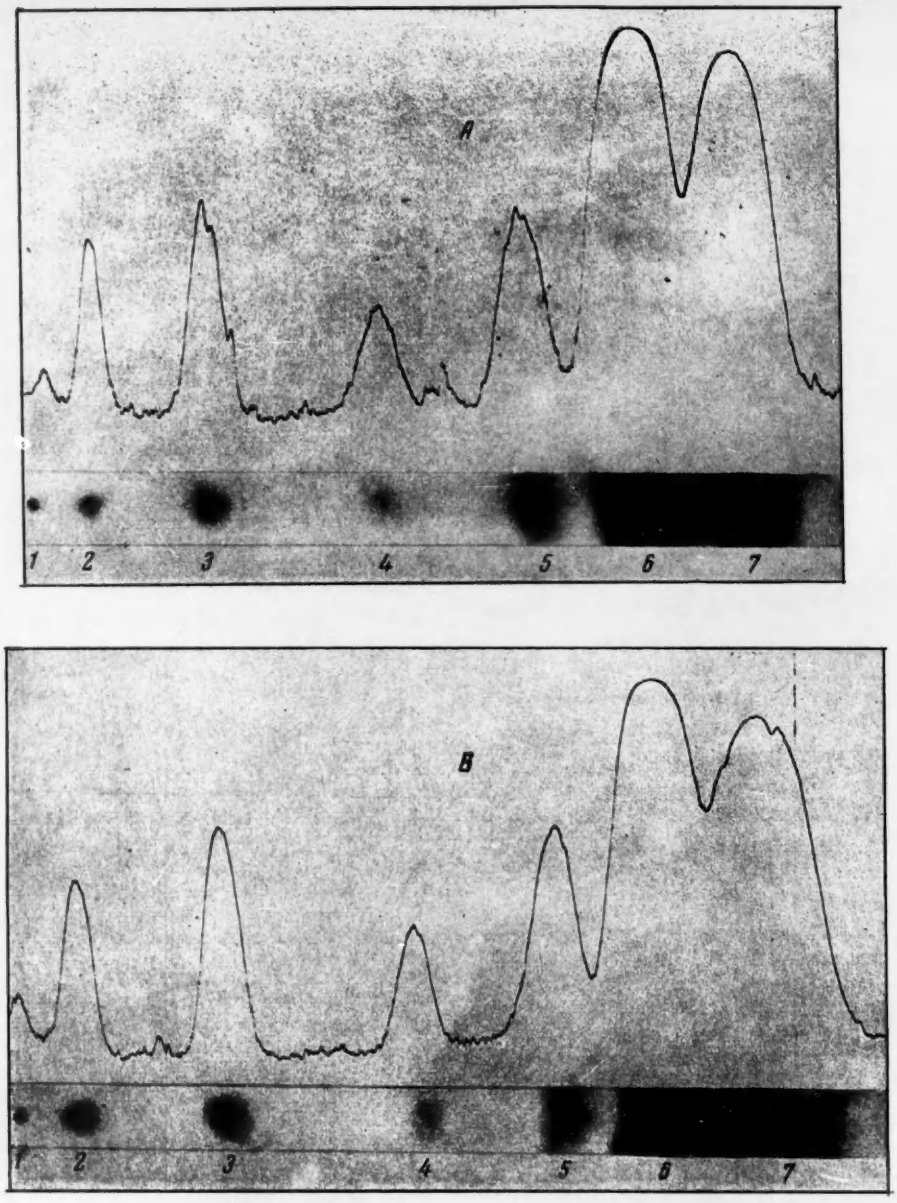

Fig. 4. Radioautographs from chromatograms of methyl esters of higher fatty acids of peanut oil and corresponding photometric curves. Solvent system: Acetic acid-acetone (3:1). Acids: 1) Lignoceric; 2) behenic; 3) arachidic; 4) stearic; 5) palmitic; 6) oleic; 7) linoleic. Deposited on the chromatogram: A) 20.0 μg; B) 29.8 μg.

The size of the recording photographic plate in the MF-4 microphotometer was 13-18 cm. Therefore, if the darkening on the radioautograph corresponding to the methyl esters of the higher fatty acids occupied a portion greater than 16.5 cm, photometry was carried out on two, and in some cases, on three plates.

Identification of the separate fractions of methyl esters of the higher fatty acids on the radioautograph was carried out by comparison with radioautographs of the methyl esters of individual higher fatty acids. In running this study we maintained identical experimental conditions. On the basis of the record on the plate we could estimate the optical density of the different portions of the radioautograph and after mathematical treatment of the results we could calculate the percent ratio and absolute amount of separate fractions of higher fatty acids in the mixtures studied.

We measured the area on the photographic plate, by a planimeter; the area bounded by the curves corresponding to the separate fractions of methyl esters of the higher fatty acids (S_1, S_2, $S_3 \ldots S_n$) on each chromatogram. The results of the measurements were multiplied by the molecular weights of the corresponding higher fatty acids ($M_1, M_2, M_3 \ldots M_n$). The percent content of the separate acids (K_1, K_2, $K_3 \ldots K_n$) in a given mixture was found by the following formula

$$K_x = \frac{S_x \cdot M_x \cdot 100\%}{S_1 \cdot M_1 + S_2 \cdot M_2 + S_3 \cdot M_3 \ldots S_n \cdot M_n}$$

where instead of S_x and M_x we substitute the data for the corresponding fatty acids (K_1, $K_2 \ldots K_n$).

The absolute quantity of separate higher fatty acids in the mixtures studied was determined by the formula: $K_x = N \cdot K_x/100$ in %, where N is the amount of higher fatty acid in μg deposited on a given chromatogram; K_x% the percent content of the corresponding higher fatty acid in the studied mixture.

Results of Tests of the Method of Quantitative Chromatography of Higher Fatty Acids

Artifically prepared mixtures of higher fatty acids were methylated with radioactive diazomethane and 39 μg of the mixture with activity of 830 counts/min was separated chromatographically in the system methanol-acetone (3:1) for 19 hours. The chromatogram was kept in contact with the X-ray film for a week. The radioautograph was treated photometrically. By considering the corresponding photometric curves, we obtained the following results (Figures in parentheses are the percentages):

Higher fatty acids	Used for chromatographic study, μg	Experimental findings, μg
Palmitic	2,4 (6,1)	2,5 (6,4)
Oleic	9,5 (24,4)	9,7 (24,9)
Linoleic	18,1 (46,3)	8,2 (46,7)
Myristic	5,7 (14,6)	5,5 (14,1)
Lignoceric	3,3 (8,5)	3,1 (7,9)

In the next experiment methylation with diazomethane-C^{14} was carried out on a mixture of lignoceric, behenic, palmitic, oleic, and linoleic acids. The radioautograph and photometric curve are shown in Fig. 2. Analysis of the results gave the following (figures in parentheses are the percentages):

Higher fatty acids	Used for chromatographic study, μg	Experimental findings, μg	R_f of methyl esters in acetic acid-acetone (3:1)
Lignoceric	0,54 (2,16)	0,51 (2,25)	0,08
Behenic	0,78 (3,00)	0,75 (2,99)	0,18
Palmitic	2,60 (10,40)	2,63 (10,54)	0,47
Oleic	8,41 (33,64)	8,39 (33,54)	0,57
Linoleic	12,70 (50,80)	12,67 (50,68)	0,67

The data on chromatographic separation of the mixture of methyl esters of the higher fatty acids shows a sufficiently high accuracy and separating power for the suggested method.

This method of quantitative chromatography was tested in the analysis of higher fatty acids of plant oils from castor and peanut seeds. In Fig. 3 we give the radioautograph and corresponding photometric curve for the chromatographic separation of the methyl esters ($-C^{14}H_3$) of the higher fatty acids of castor oil (we used 24.3 μg for the analysis). From the results of planimetric study of the photometric curve we calculated the percentages of the separate acids in the oil. Comparison of our data with the literature gives the following:

Higher fatty acid	Our data	Literature data [9]	Literature data [10]
Stearic	2,9	3	—
Oleic	5,2	3—9	7
Linoleic	6,1	2—3	3—4
Ricinoleic	85,8	80—88	87—88

Thus, the results of the analysis by our method of paper chromatography agree with the results obtained by chemical methods of macroanalysis of the mixture of fatty acids [9, 10].

In an analogous way we determined the percent content of the separate acids in a sample of peanut oil (Fig. 4). In this analysis, beginning with the step of extraction by ether we ran through the procedure again. The data show the good reproducibility of the results (Table).

TABLE

Analysis of Higher Fatty Acids of Peanut Oil Deposited on Chromatogram, 20-30 μg

Higher fatty acids	% Content in determination		R_f of methyl in system acetic acid-acetone (3:1)
	A	B	
Lignoceric	0,16	0,21	0,03
Behenic	0,63	0,73	0,08
Arachidic	1,25	1,29	0,19
Stearic	0,73	0,75	0,35
Palmitic	11,49	12,36	0,48
Oleic	56,49	51,51*	0,57
Linoleic	33,41	29,18*	0,65

* The data on content of oleic and linoleic acids was made more accurate in these samples by running the chromatographic analysis with the least amount of higher fatty acids of peanut oil (3-5 μg).

These results on the content of oleic, linoleic, and saturated higher fatty acids in peanut oil correspond to the results of macrochemical analysis [10].

This method can also be used for analysis of higher fatty acids labeled with radioactive isotopes. In this case, part of the acid mixture is methylated with diazomethane-C^{14} and another portion of the acids is methylated with nonradioactive diazomethane. By comparing the corresponding radioautographs of chromatographic separation of methyl esters of higher fatty acids, it is

possible to calculate both the percent content and the distribution of the radioactive marker in the different higher fatty acids.

SUMMARY

The proposed method is for quantitative chromatography of higher fatty acids in the form of methyl esters ($RCOOC^{14}H_3$) on hydrophobic paper followed by radioautography of the chromatogram and planimetric treatmen of the photometric curves. Methylation of the higher fatty acid is carried out with diazomethane ($C^{14}H_2N_2$).

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A CONTROL APPARATUS FOR COLUMN CHROMATOGRAPHY

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The existing types of fractional collectors and also the systems of columns and apparatus used for migration ans supplying ion exchangers (supporting media) with eluting liquids or chromatographic solvents so not always assure the desired results and do not exclude the possibility of accidents which may involve unfavorable consequences when using large volumes of alkali or easily inflammable eluants. This is because the column and the eluting system are not connected with the fractional collector as a total unit so that they function almost in parallel, bu independently of each other. On the basis of the gravimetric fractional collector of the firm of Shandon we have worked out a control apparatus which permits, first, connection of the work of the column and the fractional collector in one system, which in itself leads to a minimum chance of accident, and second, permits better work of the column and its regulation.

The collector of the firm of Shandon was modified as follows: The mercury contact set on the balance arm to make the electric circuit when the container is filled to an established weight was replaced by a photoelectric apparatus, since it easily got out of order due to rapid oxidation of the opern mercury surface when an electric spark occurred. On the central column a photoresistance FS-K2 was securely fastened and a screened light source. Between these we placed a light opaque screen, fixed on a movable balance arm in such a way that in the idle position (during the process of filling the alternate container on the weighing platform) this screen darkened the photoresistance and when the balance arm dropped under the weight of the filled container, the screen dropped along with it. As a result, a ray of light fell on the photoresistance and a relay conveyed a signal to the motor circuit.

In the work of the control apparatus we provided for the following possible breakages: stoppage for any reason of the supply of solvent in the column, any sort of break in the collector, shutting off the electectricity. In all these cases the supply of eluting liquid or chromatographic solvent was automatically stopped and the collector was cut off from the electrical circuit. The first of these operations was carried out with a needle valve with an electromagnetic valve. This device (Fig. 1) has a double function: an automatic shut-off which stopped the supply of eluting liquid or solvent in the column and a regulator of the rate of flow of the solvent through the ion exchange material (supporting medium). Regulation of the rate was carried out by control of the entrance of air in the upper reservoir with the eluting liquid (solvent), while the adjustment of the needle valve permitted this control with an accuracy and ease imposssible when the rate of outflow was regulated by a lower valve on the column.

This type of systems permits avoidance of a column with a lower capillary valve and simplifies the construction. Also, in working with this method it is easy to avoid evaporation of the eluting liquid or solvent and to create a closed system. This permits, first of all, working with a large volume of volatile liquid, and second, assures work under sterile conditions. Since when the regime of the needle valve is in operation there is an outflow, full covering of the system by use of the electromagnetic valve very quickly creates a vacuum in the upper reservoir which stops the entry of the liquid into the column. It is important that the lower end of the capillary tube which is located in the upper reservoir and is connected with the needle valve should be placed directly over the bottom of the reservoir. Such a position of the capillary assures an even rate of outflow of the liquid independent of the level of the liquid in the vessel and the resulting hydrostatic pressure.

In order to prevent exposure of the upper layer of ion exchanger or supporting medium as a result of passage of the entire volume of eluting liquid (solvent) or any break of the connection of the column with the reservoir, we used a photoelectric device ahalogous to that described above. In the upper part of the column, as shown in Fig. 1, we fixed a light source and a photoresistance, and in the column we placed on opaque glass float. In the normal working position the float was above the light beam, but when the layer of liquid over the ion exchanger (supporting medium) dropped below a critical level, the photoresistance was screened. As a result of this a relay cut off the supply to the collector and at the same time closed the electromagnetic valve. The usual method of preventing exposure and drying of the ion exchanger (supporting medium) consists in lengthening and raising the outlet canal of the column above the level of the exchanger [1]; in spite of its simplicity, this had

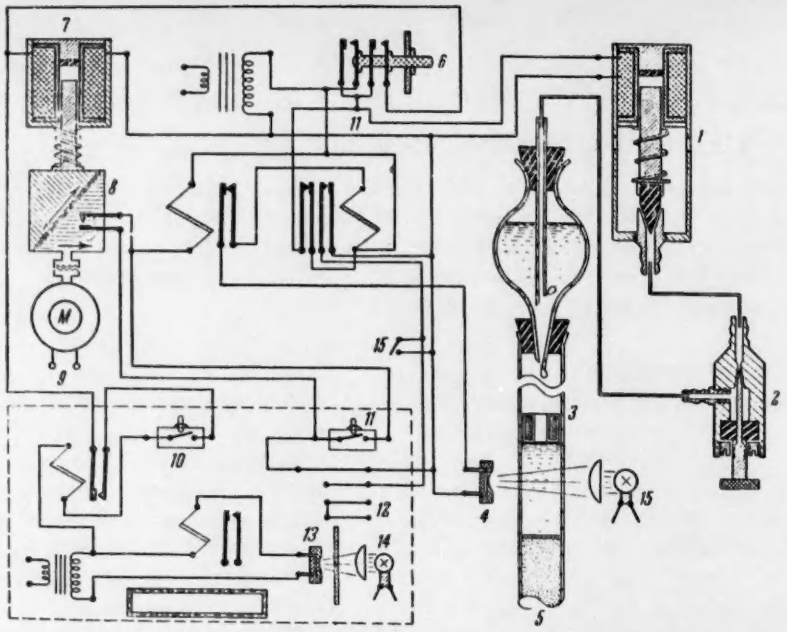

Fig. 1. Principle of the control apparatus for column chromatography (plan of the contacts shown in the working position). 1) Electromagnetic "shut-off" valve (at the time when the valve is open); 2) supply needle valve; 3) float; 4) FS-K2 photoresistance; 5) column; 6) starting button; 7) electromagnetic time control breaker; 8) timing relay; 9) connections with contacts 7 and 11 of the collector; 10) rotating microswitch; 11) microswitch for next container; 12) connection with contacts 11 and 12 of the collector; 13) FS-K2 photoresistance; 14) light source.

the difficulty that mixing of the eluate is increased and the rate of passage of the liquid through the ion exchange resin or supporting medium is slowed. In work by the method indicated, the volume in which mixing of the fractions can occur after washing is reduced to a minimum.

Since control of all possible operations and their damage in the work of the collector (and also the creation in connection with this of a complex signalling system is inexpedient, we controlled only the final result of the work of the collector, namely, the moment of shifting the rotating table and placing the empty container on the weighing platform. For this purpose the movement of the rotating table was adjusted by a timing relay in which the controlling time had been set earlier (usually 1.5-2 times longer than the average time of collecting one fraction). If the filled flask did not shift during this time interval, the relay cut off the collector and closed the electromagnetic valve, but when the collection of the fractions and shift of the collection vessels occurred normally, the timing relay each time automatically passed to the new control cycle.

As already mentioned, in all the cases considered a signal of disturbance in the work of the fraction collector or the column went to the electromagnetic valve of the automatic shut-off so that the system was closed and the supply of solvent to the column was stopped. Automatic stoppage of the elution occurred when the supply of electricity to the collector was stopped. The electrical diagrams of the addition to the fraction collector along with the control device leading to the contacts 7 and 11 of the fractional collector are shown in Fig. 1.

The system described was worked out by us in connection with work on fractionation of polymers of DNA on an Ecteola cellulose anion exchanger [2]. The prolonged character of the fractionation, which lasted 20-25 days, and also the small amount of DNA, especially DNA of virus origin and bacterial DNA with transforming ability, determined the particular needs and expectations of the apparatus (interference with the working regime usually led to loss of unique material and the results of months of work). Our design, made up of commercially available elements, was fully satisfactory and assured the desired functioning of the collector and column over a long period of continuous work.

One of the chromatographic profiles obtained on this apparatus for DNA is given in Fig. 2. In this experiment, thymus DNA with a molecular weight of 4×10^6 in the amount of 2 mg was placed on a column which contained 0.5 g of exchanger, and was eluted by the system of solvents shown in the figure. Fractionation

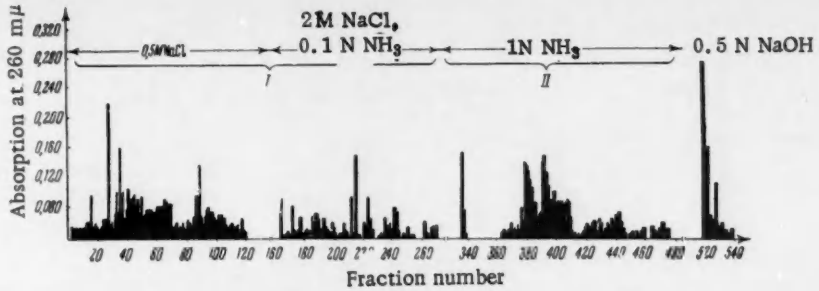

Fig. 2. Chromatographic profile of DNA. 1) Phosphate buffer, 0.01 M, pH 7; II) 2 M NaCl.

lasted 21 days at 8-10° with a rate of 5-6 ml/hour. The resulting chromatographic profile reflects the separation of the DNA into 540 collected fractions.

We will not consider here the details of the method used and the results obtained, noting only that our data agree with the results of Bendich et al., [3] who studied an analogous DNA heterogeneity. The resulting profile is sufficiently well reproducible, and the DNA peaks in repeated chromatography were eluted by solvents of the same concentration and pH as in the first chromatographic cycle. This indicates that the observed differential elution of DNA most probably depends on differences in chemical composition, structure, and molecular weight of the DNA, and not on degradation or denaturative changes of the DNA in the separation of the preparation or its chromatography. At the same time, there is no doubt that partial depolymerization of the DNA preparation to a definite degree alters the original distribution of the DNA molecules in the fractions.

SUMMARY

We have described a system of a control apparatus which permits combining into one system the work of the fractional collector, the column, and the eluting apparatus. We have given a chromatographic profile of thymus DNA obtained by fractionation of a polymeric preparation on an anion exchanger using the system which we described.

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BOOK REVIEW

PAPER CHROMATOGRAPHY. EDITED BY I. HAIS AND K. MACEK. 2ND EDITION. CZECHOSLOVAK ACADEMY OF SCIENCES PRESS, PRAGUE, 1959

(Translated from Biokhimiya Vol. 25, No. 2, pp. 380-381, March-April, 1960)

The contemporary development of biological chemistry depends to a considerable extent on the development and introduction of new methods of investigation. One of the most widely useful and dependable advances in a number of branches of theoretical and applied biochemistry is the method of chromatography. The literature on the theoretical basis and especially on the use of this method for the solution of extremely varied problems in studies of composition and metabolismin of different living objects, animals, plants, and microorganisms in vast and numerous. Therefore the systematization and generalization of this literature can be carried out only by a collective authorship, where each author is a specialist on a definite and concrete problem.

The monograph Paper Chromatography is a textbook written by a group of twenty scientific workers who work in various research institutions and higher schools of Czechoslovakia. It is favorably distinguished from the first edition (1954) by the addition of the newer literature, including all the work to the end of 1955 and some 1956 work, although the general review of the literature has been considerably shortened by exclusion of the older work from it.

The monograph begins with a historical introduction (written by I.M. Hais) and considers the theoretical principles of chromatography with particular attention to paper chromatography (chaper written by A. Tokstein, I. Dvoak, and I.M. Hais). There is a detailed discussion of the question of preparation of solutions to be studied and their deposition on the paper (I.M. Hais), the problem of the choice of the paper (R. Konop, K. Macek, and I.M. Hais), and the preparation of various kinds of modified paper (oxidized cellulose, deposition on the paper of a fine layer of various ion exchange resins, preparation of cellulose derivatives with different ionizing groups, impregnation of the paper for its use as a bearer of lyophilic groups, acetylation of the paper, etc.). There is a detailed description of the choice of solvents (K. Macek and J. Prochazka), the technique of chromatography (K. Macek and I.M. Hais), the effect of various factors on the R_f values, and problems of the reproducibility of these R_f values (I.M. Hais), and also various indicators (I.M. Hais). Special chapters are devoted to questions of detecting the separation of compounds on the chromatogram (I.M. Hais) and methods of their quantitative determination (I.M. Hais and P. Zuman).

The problems of paper chromatography of radioactive substances are considered (I.M. Hais). A separate chapter is devoted to methods of preparative paper chromatography, and also of columns of paper layers (J. Prochaska and M. Kutacek). Special chapters of the monograph are devoted to methods of paper chromatography of special classes of organic compounds: alcohols (M. Macek), aldehydes and ketones (K. Macek), aliphatic acids (K. Macek and C. Michalec), sugars (K. Macek), phenols and aromatic acids (J. Prochazka), steroids (O. Siblikova), carbohydrates and other lipophlic compounds (O. Silbikova), aliphatic and aromatic amines (J. Gasparic), polyamides and their constituent parts (K. Macek), organic nitro compounds (J. Franz), amino acids (I. M. Hais), peptides and problems of study of protein structure (B. Meloun), separation of proteins, including enzymes (K. Macek), purines, pyrimidines a and nitrogenous parts of nucleic acids (Z. Padr), alkaloids (K. Macek), other nitrogenous heterocyclic, purine derivatives (K. Macek), indole derivatives (J. Prochazka), imidazole derivatives (A. Jenicek), pyrrole derivatives (A. Komarkova), plant leaf pigments (C. Michalec), other compounds (K. Macek), organic sulfur-containing compounds (I. Gasparic), vitamins (I.M. Hais) including pterine derivatives (K. Slavik), vitamins of the B_{12} group (V. Rabek), ascorbic acid and related compounds (J. Prochazka), and also antibiotics (M. Donal and I.M. Hais), insecticides (V. Batora), synthetic dyes (J. Franz). A separate chapter discusses the paper chromatography of inorganic compounds. (B. Kakac).

Two additional chapters of the book (written by K. Macek) are devoted to a description of indicating reagents and their preparation, and also the preparation of samples to be studied; The composition of solution for impregnating the paper are described as are the preparation of solvents and examples of quantitative analysis. This description is a supplement to each individual chapter of the monograph, in which these reagents are only mentioned, but the method of their preparation is not given.

The book is provided with an author and subject index and is very easy to use. It is well illustrated with figures, diagrams, tables, photographs, including color photographs of chromatograms. The diagrams of chro-

matograms are especially good, showing whether various compounds are separated in the different solvents used. These diagrams are much more graphic than the tables with their calculated R_f values for different compounds in given systems of solvents.

The monograph Paper Chromatography , written by a group of CZech investigators, deserves the gratitude of biochemists. The first edition of this book was translated into German. The monograph is the most complete current textbook and can be recommended as a reference work for every laboratory in which studied are carried out using the method of paper chromatography.

Z. S. Kagan and E. Galyas

SCIENCE NEWS

PLAN OF CONGRESS

(Translated from Biokhimiya Vol. 25, No. 2, p. 382, March-April, 1960)

The scientific program of the Fifth International Biochemical Congress to be held in Moscow August 10-16, 1961 provides for the work of eight symposia on basic problems of contemporary biochemistry, a description of which and also a list of the questions with which they will be concerned was published earlier in the journal "Biokhimiya".

According to the program, the symposia will have four sessions lasting three hours each. It is proposed that each session of the symposia will hear one main address, 5-7 formal papers, and will also have a free discussion. In accordance with this distribution of time the scientific program of the symposia has been arranged for the discussion of four basic themes in each problem, so that each session of a symposium will be devoted to one theme.

At the present time the presidents of the symposia, with the organizers chosen by the Organizing Committee from a number of Soviet scientists, are working out detailed programs for each session of the symposia, discussing this with the individual participants in the symposia, and compiling the actual working program.

For the information of the public we give the planned program of two of the symposia.

Symposium No. 6

Mechanism of photosynthesis. President – H. Tamiya (Japan), organizer – A.A. Krasnovskii (USSR).

The following basic themes are planned for this symposium:

1. Photochemistry and spectroscopy of pigment systems in photosynthetic organisms. First address on the theme, "Photochemistry of Chlorophyll and the Mechanism of Photosynthesis" will be given by Prof. R. Wurmser (France).

The accompanying papers at this session will be by Prof. E. Rabinowitch (USA), Dr. R. Livingston (USA), and Dr. A. Terenin (USSR) and others.

2. Nature of the primary photochemical act of photosynthesis: state and transformations of pigments in the organism. First address: "Nature of the Primary Act in Photosynthesis" by Prof. E. Wassink (Holland). The accompanying papers at this session will be given by Dr.S. French (USA), Prof. L. Duisens (Holland), Dr.L. Smith (USA), N. Witt (Federal German Republic), Dr.A. Krasnovskii (USSR) and others.

3. Nature of the related photochemical, and enzymatic stages: first address "Photosynthetic Phosphorylation in Photosynthesis" will be given by Prof. D. Arnon (USA). Formal papers will be given by Prof. W. Vishniak (USA), Dr.R. Kamen (USA) and Dr. Boichenko (USSR) and others.

4. First products of the fixation and assimilation of carbon dioxide in photosynthesis. First address: "The Path of Carbon in Photosynthesis by Plants" by Prof. M. Calvin (USA). The formal papers will be given by Dr. A. Moyse (France), Prof.H. Metzner (Federal German Republic), Dr.M. Allen (USA), Dr. Fuller (USA), Dr.A. Nichiporovich (USSR) and others.

Symposium No. 8

The biochemical basis for the technology of food production. President – E. Pianowsky (Poland), organizer – V. Kretovich (USSR).

The four basic themes planned for this symposium are:

1. Biochemistry and selection. First address: "Biochemical Principles for Selection for Quality". Accompanying papers at this session by Prof.V. Schuphan (Federal German Republic), Dr. Roubaud (Belgium), Dr.V. Bukin (USSR) and others.

2. Biochemical basis for the technology of plant food sources. First address: "Amino Acid Composition of Cereals and Methods for Improving This". Papers: Prof.M. Millner (USA), Dr.A. Holm (England), Dr.R. Robertson (Australia), Dr.V. Kretovich (USSR) and others.

3. Biochemical basis for the technology of animal food sources. First address: "General Problems of the Biochemical Basis for Milk Technology", given by Prof. Key (England). Papers: Prof.H. Fink (Federal German Republic), O. Hannan (England), Dr.R. Grau (Federal German Republic) Dr. B. Watts (USA), P. D'yachenko (USSR) and others.

4. Biochemical basis for fermentation products. First address: "Biochemical Problems of Fermentation Products" given by Prof. De Clercq (Belgium). Papers: Prof.V. Trevelyan (England), L. Janitsky (Poland), Dr.L. Veselov (USSR) and others.

G. A. Deborin

NECROLOGY

In Memory of D. M. Mikhlin

(Translated from Biokhimiya Vol. 25, No. 2, p. 383, March-April, 1960)

On June 30, 1959 at the age of 72 Professor David Mikhailovich Mikhlin, Doctor of Biological Science, Director of the Laboratory of Biological Oxidation of the A.N. Bakh Institute of Biochemistry, Academy of Sciences, USSR, ended this services as a scientific worker for the RSFSR.

Biochemistry has lost a great specialist, very learned in the field of biological oxidations. The best chemical and biological training (he completed the physicomathematical and medical courses at Moscow University), and a great love for science assured for D.M. Mikhlin a successful career in those fields of biochemistry which include chemistry and biology.

He began his investigations with a study of the Schardinger enzyme - aldehyde or purine oxidase - in 1921 in the Institute of Biochemistry of the Ministry of Health under the direction of A.N. Bakh. As a result of a study over many years of the mechanism of action of this enzyme, D.M. Mikhlin showed that its preparations, which had previously been considered to be single, actually consisted of two enzymes, aldehyde oxidase and aldehydemutase. Study of these enzymes led D.M. Mikhlin to the discovery of the enzymatic nature of reduction of nitrates to nitrites in green plant cells. He succeeded in isolating from the green cells a preparation which could carry out the characteristic aldehyde oxidase reaction by reduction of nitrates to nitrites. Then he showed the ability of plants to reduce nitrites to hydroxylamine. D. M. Mikhlin observed this in potatoes.

An important place in the investigations of D. M. Mikhlin was occupied by questions of distribution, localization, and mechanism of action of various plant oxidases. He showed that in the early stages in the development of plants (barley, wheat, kokh-sagyz) the final step in the oxidation was carried out by the cytochrome oxidase system, which in the latter stages of development yielded its place to other oxidizing systems.

In studying the mechanism of action of plant lipoxidases, D.M. Mikhlin showed for the first time that the lipoxidase substrates filled the role of the "oxygenase" of A.N. Bakh and that the lipoxidase system, activating oxygen, shared in plant respiration in combination with other oxidizing systems especially with peroxidase and polyphenol oxidase systems.

Besides the study of biological oxidations, in the course of his studies D.M. Mikhlin also undertook the investigation of other problems of biochemistry. Thus, for example, he studied the enzymes which took part in transformations of carbohydrates, fats, and organic acids. He first obtained experimental evidence of the synthesis of citric acid from oxaloacetic and pyruvic or malic acids in plants and first isolated the natural antihemorrhagic factor from butter which sharply raised the coagulating power of normal blood.

D.M. Mikhlin also undertook the study of plant tannins, the path of formation of rubber in plants, and other questions.

D.M. Mikhlin published 85 scientific papers, mostly in the field of biological oxidations, including review papers in this number. He write two monographs, "Peroxides and Peroxidases" (for which he received the A.N. Bakh Prize), and "Biological Oxidations". His third monograph, "Biochemistry of Cell Respiration", on which he worked until his last days, is in press.

D.M. Mikhlin trained a corps of biochemists every day. He was Docent at Moscow University, held the chair of biochemistry at the Ivanov Medical Institute and the Moscow Pharmaceutical Institute. He trained a number of candidates and doctors in science, who have worked in various scientific institutes.

Duringhis whole life, D.M. Mikhlin gave disinterested and devoted service to science. This was the basic position which he held and which at once led his students and followers to a deepened study of the processes of biological oxidation.

P.A. Kolesnikov.

For the Information of Readers

The Press of the Academy of Sciences, USSR, each year publishes more than 800 books on social, physico-mathematical, chemical, geologic-geographic, and biological sciences, technology, industry, transport, and questions of agriculture.

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RUSSIAN JOURNALS FREQUENTLY CITED
[Biological Sciences]

Abbreviation*	Journal*	Translation
Agrobiol.	Agrobiologiya	Agrobiology
Akusherstvo i Ginekol.	Akusherstvo i Ginekologiya	Obstetrics and Gynecology
Antibiotiki	Antibiotiki	Antibiotics
Aptechnoe Delo	Aptechnoe Delo	Pharmaceutical Transactions
Arkh. Anat. Gistol. i Embriol.	Arkhiv Anatomii Gistologii i Embriologii	Archives of Antomy, Histology, and Embryology
Arkh. Biol. Nauk SSSR	Arkhiv Biologicheskikh Nauk SSSR	Archives of Biological Science USSR
Arkh. Patol.	Arkhiv Patologii	Archives of Pathology
Biofizika	Biofizika	Biophysics
Biokhimiya	Biokhimiya	Biochemistry
Biokhim. Plodov i Ovoshchei	Biokhimiya Plodov i Ovoshchei	Biochemistry of Fruits and Vegetables
Bot. Zhur.	Botanicheskii Zhurnal	Journal of Botany
Byull. Eksptl. Biol. i Med.	Byulleten Eksperimentalnoi Biologii i Meditsiny	Bulletin of Experimental Biology and Medicine
Byull. Moskov. Obshchestva Ispytatelei Prirody, Otdel Biol.	Byulleten Moskovskogo Obshchestva Ispytatelei Prirody, Otdel Biologicheskii	Bulletin of the Moscow Naturalists Society, Division of Biology
Doklady Akad. Nauk SSSR	Doklady Akademii Nauk SSSR	Proceedings of the Academy of Sciences USSR
Éksptl. Khirurg.	Eksperimentalnaya Khirurgiya	Experimental Surgery
Farmakol. i Toksikol.	Farmakologiya i Toksikologiya	Pharmacology and Toxicology
Farmatsiya	Farmatsiya	Pharmacy
Fiziol. Rastenii	Fiziologiya Rastenii	Plant Physiology
Fiziol. Zhur. SSSR	Fiziologicheskii Zhurnal SSSR im. I. M. Sechenova	I. M. Sechenov Physiology Journal USSR
Gigiena i Sanit.	Gigiena i Sanitariya	Hygiene and Sanitation
Izvest. Akad. Nauk SSSR, Ser. Biol.	Izvestiya Akademii Nauk SSSR, Seriya Biologicheskaya	Bulletin of the Academy of Sciences USSR, Biology Series
Izvest. Tikhookeanskogo N. I. Inst. Rybnogo Khoz. i Okeanog.	Investiya Tikhookeanskogo N. I. Instituta Rybnogo Khozyaistva i Okeanografii	Bulletin of the Pacific Ocean Scientific Institute of Fisheries and Oceanography
Khirurgiya	Khirurgiya	Surgery
Klin. Med.	Klinicheskaya Meditsina	Clinical Medicine
Lab. Delo	Laboratornoe Delo (po Voprosam Meditsiny)	Laboratory Work (on Medical Problems)
Med. Parazitol.	Meditsinskaya Parazitologiya i Parazitarnye Bolezni	Medical Parasitology and Parasitic Diseases
Med. Radiol.	Meditsinskaya Radiologiya	Medical Radiology
Med. Zhur. Ukrain.	Medichnii Zhurnal Ukrainskii	Ukrainian Medical Journal
Mikrobiologiya	Mikrobiologiya	Microbiology
Mikrobiol. Zhur.	Mikrobiologichnii Zhurnal	Microbiology Journal
Nevropatol., Psikhyat. i Psikhogig.	Nevropatologiya, Psikhyatriya i Psikhogigiena	Neuropathology, Psychiatry and Psychohygiene
Ortoped., Travmatol. i Protez.	Ortopediya, Travmatologiya i Protezirovanie	Orthopedics, Traumatology and Prosthetics
Parazitol. Sbornik	Parazitologicheskii Sbornik	Parasitology Collection
Pediatriya	Pediatriya	Pediatrics
Pochvovedenie	Pochvovedenie	Soil Science
Priroda	Priroda	Nature
Problemy Endokrinol. i Gormonoterap.	Problemy Endokrinologii i Gormonoterapii	Problems of Endocrinology and Hormone Therapy
Problemy Gematol.	Problemy Gematologii i Perelivaniya Krovi	Problems of Hematology and Blood Transfusion
Problemy Tuberk.	Problemy Tuberkuleza	Problems of Tuberculosis
Sovet. Med.	Sovetskaya Meditsina	Soviet Medicine
Sovet. Vrachebny Zhur.	Sovetskii Vrachebnyi Zhurnal	Soviet Physicians Journal
Stomatologiya	Stomatologiya	Stomatology

*BRITISH-AMERICAN TRANSLITERATION SYSTEM.

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Abbreviation	Journal	Translation
Terap. Arkh.	Terapevticheskii Arkhiv	Therapeutic Archives
Trudy Gelmint. Lab.	Trudy Gelmintologicheskoi Laboratoriya	Transactions of the Helminthology Laboratory
Trudy Inst. Genet.	Trudy Instituta Genetiki	Transactions of the Institute of Genetics
Trudy Inst. Gidrobiol.	Trudy Instituta Gidrobiologiya	Transactions of the Institute of Hydrobiology
Trudy Inst. Mikrobiol.	Trudy Instituta Mikrobiologiya	Transactions of the Institute of Microbiology
Trudy Inst. Okean.	Trudy Instituta Okeanologiya, Akademii Nauk SSSR	Transactions of the Institute of Oceanology, Academy of Sciences, USSR
Trudy Leningrad Obshchestva Estestvoisp.	Trudy Leningrad Obshchestva Estestvoispytatelei	Transactions of the Leningrad Society of Naturalists
Trudy Vsesoyuz. Gidrobiol. Obshchestva	Trudy Vsesoyuznogo Gidrobiologicheskogo Obshchestva	Transactions of the All-Union Hydrobiological Society
Trudy Vsesoyuz. Inst. Eksptl. Med.	Trudy Vsesoyuznogo Instituta Eksperimentalnoi Meditsiny	Transactions of the All-Union Institute of Experimental Medicine
Ukrain. Biokhim. Zhur.	Ukrainskii Biokhimichnii Zhurnal	Ukrainian Biochemical Journal
Urologiya	Urologiya	Urology
Uspekhi Biokhimiya	Uspekhi Biokhimiya	Progress in Biochemistry
Uspekhi Sovremennoi Biol.	Uspekhi Sovremennoi Biologiya	Progress in Contemporary Biology
Vestnik Akad. Med. Nauk SSSR	Vestnik Akademii Meditsinskikh Nauk SSSR	Bulletin of the Academy of Medical Science USSR
Vestnik Khirurg. im. Grekova	Vestnik Khirurgii imeni Grekova	Grekov Bulletin of Surgery
Vestnik Leningrad. Univ. Ser. Biol.	Vestnik Leningradskogo Universiteta, Seriya Biologii	Journal of the Leningrad Univ., Biology Series
Vestnik Moskov. Univ., Ser. Biol. i Pochvov.	Vestnik Moskovskogo Universitera, Seriya Biologii i Pochvovedeniya	Bulletin of the Moscow University, Biology and Soil Science Series
Vestnik Oftalmol.	Vestnik Oftalmologii	Bulletin of Ophthalmology
Vestnik Oto-rino-laringol.	Vestnik Oto-rino-laringologiya	Bulletin of Otorhinolaryngology
Vestnik Rentgenol. i Radiol.	Vestnik Rentgenologii i Radiologii	Bulletin of Roentgenology and Radiology
Vestnik Venerol. i Dermatol.	Vestnik Venerologii i Dermatologii	Bulletin of Venereology and Dermatology
Veterinariya	Veterinariya	Veterinary Science
Vinodelie i Vinogradarstvo	Vinodelie i Vinogradarstvo SSSR	Wine-Making and Viticulture
Voprosy Klin.	Voprosy Klinicheskie	Clinical Problems
Voprosy Med. Khim.	Voprosy Meditsinskoi Khimii	Problems of Medical Chemistry
Voprosy Med. Virusol.	Voprosy Meditsinskoi Virusologii	Problems of Medical Virology
Voprosy Neirokhirurg.	Voprosy Neirokhirugii	Problems of Neurosurgery
Voprosy Onkol.	Voprosy Onkologii	Problems of Oncology
Voprosy Pitaniya	Voprosy Pitaniya	Problems of Nutrition
Voprosy Psikhologii	Voprosy Psikhologii	Problems of Psychology
Voprosy Virusologii	Voprosy Virusologii	Problems of Virology
Vrachebnoe Delo	Vrachebnoe Delo	Medical Profession
Zav. Lab.	Zavodskaya Laboratoriya	Factory Laboratory
Zhur. Mikrobiol., Epidemiol. i Immunobiol.	Zhurnal Mikrobiologii, Epidemiologii i Immunobiologii	Journal of Microbiology, Epidemiology, and Immunobiology
Zhur. Nevropatol. i Psikhiat.	Zhurnal Nevropatologii i Psikhiatrii imeni S. S. Korsakov	S. S. Korsakov Journal of Neuropathology and Psychiatry
Zhur. Obshchei Biol.	Zhurnal Obshchei Biologiya	Journal of General Biology
Zhur. Vysshei Nerv. Deyatel.	Zhurnal Vysshei Nervnoi Deyatelnosti imeni I. P. Pavlova	I. P. Pavlov Journal of Higher Nervous Activity
Zool. Zhur.	Zoologicheskii Zhurnal	Journal of Zoology

SIGNIFICANCE OF ABBREVIATIONS MOST FREQUENTLY ENCOUNTERED IN SOVIET PERIODICALS

FIAN	Phys. Inst. Acad. Sci. USSR.
GDI	Water Power Inst.
GITI	State Sci.-Tech. Press
GITTL	State Tech. and Theor. Lit. Press
GONTI	State United Sci.-Tech. Press
Gosenergoizdat	State Power Press
Goskhimizdat	State Chem. Press
GOST	All-Union State Standard
GTTI	State Tech. and Theor. Lit. Press
IL	Foreign Lit. Press
ISN (Izd. Sov. Nauk)	Soviet Science Press
Izd. AN SSSR	Acad. Sci. USSR Press
Izd. MGU	Moscow State Univ. Press
LEIIZhT	Leningrad Power Inst. of Railroad Engineering
LET	Leningrad Elec. Engr. School
LETI	Leningrad Electrotechnical Inst.
LETIIZhT	Leningrad Electrical Engineering Research Inst. of Railroad Engr.
Mashgiz	State Sci.-Tech. Press for Machine Construction Lit.
MEP	Ministry of Electrical Industry
MES	Ministry of Electrical Power Plants
MESEP	Ministry of Electrical Power Plants and the Electrical Industry
MGU	Moscow State Univ.
MKhTI	Moscow Inst. Chem. Tech.
MOPI	Moscow Regional Pedagogical Inst.
MSP	Ministry of Industrial Construction
NII ZVUKSZAPIOI	Scientific Research Inst. of Sound Recording
NIKFI	Sci. Inst. of Modern Motion Picture Photography
ONTI	United Sci.-Tech. Press
OTI	Division of Technical Information
OTN	Div. Tech. Sci.
Stroiizdat	Construction Press
TOE	Association of Power Engineers
TsKTI	Central Research Inst. for Boilers and Turbines
TsNIEL	Central Scientific Research Elec. Engr. Lab.
TsNIEL-MES	Central Scientific Research Elec. Engr. Lab.—Ministry of Electric Power Plants
TsVTI	Central Office of Economic Information
UF	Ural Branch
VIESKh	All-Union Inst. of Rural Elec. Power Stations
VNIIM	All-Union Scientific Research Inst. of Meteorology
VNIIZhDT	All-Union Scientific Research Inst. of Railroad Engineering
VTI	All-Union Thermotech. Inst.
VZEI	All-Union Power Correspondence Inst.

Note: Abbreviations not on this list and not explained in the translation have been transliterated, no further information about their significance being available to us. — Publisher.

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